Effects of Octadecaenoic Acids and Apple Polyphenols on Blood Cholesterol

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Abstract

Hypercholesterolemia accelerates the progression of atherosclerosis, the early stage of many cardiovascular diseases. Octadecaenoic acids and polyphenols are commonly consumed as functional foods to control blood cholesterol. However, information on the efficacy and underlying mechanism of their hypocholesterolemic activity is currently limited.

The objectives of the present study were (i) to investigate the differential effects of four structurally similar octadecaenoic acids, namely linoleic acid (LA), conjugated linoleic acid (CLA), linolenic acid (LN) and conjugated linolenic acid (CLN) on blood cholesterol in hamster, (ii) to study the effects LA, CLA and LN on cholesterol-regulating genes in HepG2, and (iii) to investigate the effect of apple polyphenols (AP) on blood cholesterol in hamsters.

The results showed that LA, CLA, LN but not CLN significantly lowered blood total cholesterol in hamsters. These fatty acids had no effect on blood high-density-lipoprotein (HDL) cholesterol but LA, CLA and LN but not CLN reduced non-HDL cholesterol, affecting the blood lipid profile favorably. CLA and LN were found to reduce liver cholesterol more potently than LA and CLN, but Western blotting demonstrated that these four fatty acids had no effect on the sterol regulatory element-binding protein-2 (SREBP-2), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), low-density-lipoprotein receptor (LDLR), liver-X-receptor (LXR) and cholesterol-7α-hydroxylase (CYP7A1), and real-time PCR showed no effect on the mRNA of cholesterol ester transfer protein (CETP), LDLR, LXR, and CYP7A1.

However, it was found that fecal neutral output was increased by CLA and LN, but not LA and CLN. Correspondingly, measurement of the intestinal acyl cholesterol: acyl transferease (ACAT) activity showed decreased ACAT activity in hamsters fed CLA and LN but not LA and CLN, suggesting that the stronger hypocholesterolemic activity of CLA and LN over LA and CLN was mediated by their effect on intestinal cholesterol absorption via inhibition on intestinal ACAT activity.

CLA but not LA and LN significantly increased the mRNA level of SREBP-2, HMGR, LDLR, LXR, and CYP7A1 in HepG2 cells. Interestingly, CLA only significantly increased the protein level of LXR but not that of SREBP-2, LDLR and CYP7A1 under the current experimental condition. This suggested CLA had a distinctive effect on SREBP-2, HMGR, LDLR, and CYP7A1 gene expression *in vitro*, compared with LA and LN.

Supplementation of 0.3 and 0.6% of AP in diet had no significant effect on the blood total cholesterol in hamsters, but it decreased the non-HDL-cholesterol to HDL-cholesterol ratio and blood triglyceride. AP supplementation at both concentrations did not significantly affect liver cholesterol level, and had no effect on the protein level of SREBP-2, HMGR, LDLR and CYP7A1 in the liver, and CETP in plasma. However, plasma CETP activity in both AP fed groups was significantly lower than the control, leading to a lower ratio of non-HDL-cholesterol to HDL-cholesterol in AP fed hamsters. *In vitro* experiments also confirmed inhibitory effect of AP on CETP activity, suggesting that the decrease in non-HDL-cholesterol to HDL-cholesterol ratio by AP was mediated through inhibition on plasma CETP activity at a post-translational level.

In conclusion, the present study confirmed that LA, CLA and LN were effective in reducing blood total cholesterol, while AP was effective in lowering non-HDL-cholesterol to HDL-cholesterol ratio. CLA and LN significantly reduced intestinal ACAT activity and hence cholesterol absorption *in vivo* in hamsters. In HepG2 cells, CLA had a distinctive stimulatory effect on SREBP-2, LDLR, HMGR, LXR and CYP7A1 mRNA compared with LA and LN. AP inhibited plasma CETP activity and improved blood cholesterol distribution.

摘要

動脈血管粥樣化是導致許多心血管疾病的早期病狀，而高膽固醇血症可加快動脈血管粥樣化的發展。十八碳烯酸和多酚則是常被用作控制血膽固醇的功能食品成份，但其有效性及作用機理尚未確定。

本研究的主要目的有以下三點：(i) 研究四種結構相近的十八碳烯酸，即次亞麻酸 (LA)、 共軛次亞麻酸 (CLA)、亞麻酸 (LN) 和共軛亞麻酸 (CLN)對倉鼠血膽固醇的影響；(ii) 研究 LA，CLA 和 LN 對 HepG2 細胞內膽固醇調控的影響；和 (iii) 研究蘋果多酚對倉鼠血膽固醇的影響。

實驗結果表明，LA、CLA 和 LN 有效降低倉鼠的血總膽固醇，但 CLN 則沒有此功效。此四種十八碳烯酸均對血清高密度脂蛋白膽固醇無影響，但 LA、CLA 和 LN 則減少了血清非高密度脂蛋白膽固醇，從而改善了血脂譜。CLA 和 LN 亦比 LA 和 CLN 更有效降低肝臟膽固醇，但此四種脂肪酸均對肝臟內固醇調節元件結合蛋白-2(SREBP-2)、甲基戊二酸單酞輔酶 A 還原酶 (HMGR)、低密度脂蛋白受體 (LDLR)、肝-X-受體(LXR)和膽固醇 7α-羥化酶(CYP7A1)的蛋白，和 LDLR、LXR、CYP7A1 和膽固醇酯轉移蛋白(CETP)的信使核糖核酸無影響。此外，CLA 和 LN 增加了糞便中性固醇的排出，但 LA 和 CLN 則無此作用。腸臟酸基輔酶 A:膽固醇酞基轉移酶(ACAT)活性測驗則顯示，餵飼 CLA 和 LN 的倉鼠腸臟 ACAT 活性明顯下降，而餵飼 LA 和 CLN 的倉鼠的 ACAT 活性則與對照無明顯分別。此結果提示 CLA 和 LN 較 LA 和 CLN 降低膽固醇的更強活性，可能通過 CLA 和 LN 對 ACAT 的特

有抑制作用，從而減少膽固醇之吸收作簡釋。

此外，細胞培養實驗表明，CLA 顯著提高了 HepG2 細胞內 SREBP-2, HMGR, LDLR, LXR 和 CYP7A1 的核糖核酸，但 LA 和 LN 則無此效用。CLA 亦提高了 LXR 的蛋白。這些都顯示 CLA 相比 LA 和 LN 對 SREBP-2, HMGR, LDLR, LXR 和 CYP7A1 的基因表達的獨特影響。

在飼料內加入 0.3%和 0.6%的蘋果多酚，並未對倉鼠的血清總膽固醇造成顯著改變，但此兩個蘋果多酚濃度都減少了血清非高密度脂蛋白膽固醇，同時又增加了血清高密度脂蛋白膽固醇，使兩者比例減少。此兩蘋果多酚濃度亦沒有影響肝臟 SREBP-2, HMGR, LDLR 和 CYP7A1，及血清 CETP 水平。但是，被餵飼蘋果多酚的兩組倉鼠的血清 CETP 活性則較對照明顯減少。試管實驗亦證實蘋果多酚在體外具抑制 CETP 之作用。綜合所得，蘋果多酚可能通過抑制血液 CETP，達致降低非高密度脂蛋白膽固醇和提高高密度脂蛋白膽固醇之作用。

總之，本研究証實了 LA、CLA 和 LN 對降血膽固醇之功效，亦証明了蘋果多酚可降低非高密度脂蛋白膽固醇/血清高密度脂蛋白膽固醇比例。在活體內，CLA 和 LN 通過抑制 ACAT，減少腸臟膽固醇吸收；而在試管實驗，CLA 則表現了對 SREBP-2, LDLR, HMGR, LXR 和 CYP7A1 核糖核酸的獨特促進反應。蘋果多酚則通過抑制 CETP 改善活體內膽固醇在血液脂蛋白之分佈。

List of Abbreviations

ACAT	acyl coenzyme A: cholesterol acyltransferase
ANOVA	analysis of variance
AP	apple polyphenol
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CETP	cholesterol ester transfer protein
CHD	coronary heart diseases
CLA	conjugated linoleic acid
CLN	conjugated linolenic acid
CYP	cytochrome P450
CYP7A1	cholesterol-7α-hydroxylase
DEPC	diethyl pyrocarbonate
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
EPA	eicosapentaenoic acid
GC	gas chromatography
HDL	high-density lipoprotein
HDL-C	HDL-cholesterol
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
IDL	intermediate-density lipoprotein

LA	linoleic acid
LDL	low-density lipoprotein
LDL-C	LDL-cholesterol
LN	linolenic acid
LXR	liver-X-receptor
nHDL-C	non-HDL-cholesterol
ODA	octadecaenoic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PUFAs	polyunsaturated fatty acids
RCT	reverse cholesterol transport
RNA	ribonucleic acid
RXR	retinoid-X-receptor
S.D.	standard deviation
S1P	site-1-protease
S2P	site-2-protease
SCAP	SREBP cleavage-activating protein
SDS	sodium dodecyl sulfate
SR-B1	scavenger receptor-B1
SREBP-2	sterol regulatory element-binding protein-2
TBS	tris-buffered saline
TC	total cholesterol
TG	triglyceride

TLC	thin-layer chromatography
TRL	triglyceride-rich lipoprotein
VLDL	very low-density lipoprotein

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Chapter 1
General Introduction

1.1. Introduction to Cholesterol and Its Related Diseases

1.1.1 Chemistry of cholesterol

Cholesterol was first discovered in bile stone by Chevreul in 1816. Recognized as both a lipid and a sterol, pure cholesterol is an alcohol-soluble white crystal. Its chemical structure was confirmed by Bernal (1932) using X-ray crystallography.

The chemical structure of cholesterol is shown on Figure 1.1. The core structure of cholesterol is its steroid nucleus which is built from four fused hydrocarbon rings. The steroid nucleus contains three 6-carbon and one 5-carbon rings and is relatively planar and rigid, as rotation is not allowed around the C-C bonds. A cholesterol molecule also contains two functional groups, a polar hydroxyl group and a non-polar alkyl group, making it amphipathic (Nelson & Cox, 2002).

Cholesterol is transported and stored in the form of cholesterol esters. Being insoluble in water, it is circulated in the blood in lipoproteins carriers, including high-density lipoprotein (HDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and chylomicron.

1.1.2 Physiological importance of cholesterol

The major roles of cholesterol are as an essential component in cell membranes and as a precursor of a number of hormones. Cholesterol accounts for over 10% of the total lipids in plasma membrane and in the Golgi membrane (Meisenberg & Simmons, 2006). Cholesterol weakens the Van der Waals interaction between the phospholipids tails in the bilayer and maintains fluidity of the membrane (Cooper, 1978; Petrache *et al*, 2005). On the other hand, cholesterol blocks the non-specific entry of small

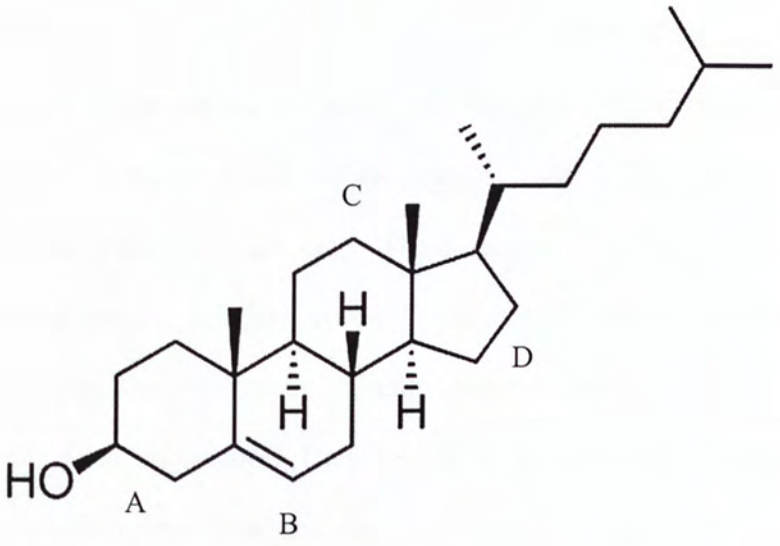

Figure 1.1

Chemical structure of cholesterol

The hydroxyl group at A ring of cholesterol is hydrophilic and the rest of the molecule is hydrophobic. A fatty acid condenses with this hydroxyl group when cholesterol is transported or stored in esterified form. The four rings of the nucleus are named A, B, C and D as shown.

molecules by its characteristic simultaneous hydrophobicity and hydrophilicity (Subczynski & Wisniewska, 2000).

Cholesterol also acts as the precursor of a number of steroid hormones, e.g. adrenocortical hormones and sex hormones. Typically, the side chain from the D ring of cholesterol is removed and the molecule is oxidized by endocrine tissues and carried in the blood to target cells. These steroid hormones couple with nuclear receptors and regulate gene expression. Some other hormones, such as progesterone, cortisol, aldosterone, testosterone and cholecalciferol (vitamin D_3) are also synthesized from cholesterol. These enzymes affect sexual development, sexual behavior, calcium deposition and mobilization and a variety of other homeostatic functions and hence are important to normal body functioning (Nelson & Cox, 2002).

1.1.3 Pathological effects of cholesterol

The most severe clinical manifestation of excessive cholesterol accumulation is atherosclerosis, which is referred to as the hardening or furring of the artery wall. Atherosclerosis of the coronary and brain artery may progress to coronary heart disease and stroke, respectively (Ross, 1993). In 2006, these two cardiovascular events together contributed to 24.1% of total mortality in Hong Kong (Department of Health, Hong Kong, 2007).

1.1.3.1 Mechanism of atherosclerosis

The development of atherosclerosis is slow and progressive. Progression of the disease from onset to symptomatic stage may last for years (Figure 1.2). Traditionally, atherosclerosis had been simply considered as the gradual accumulation of fatty substances on the wall of blood vessels, and is equivalent to the building up rust in

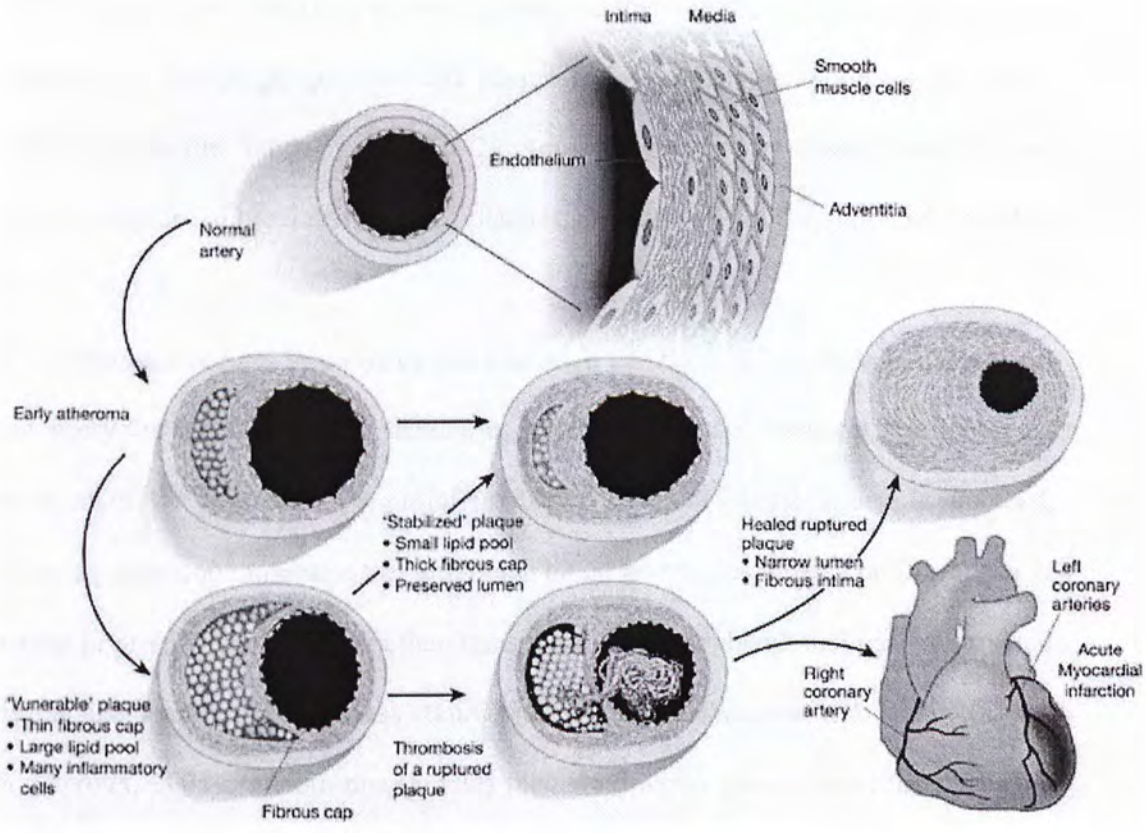

Figure 1.2

Schematic diagram on the progression of coronary atherosclerosis

Fatty streak build-up results in the formation of plaques. When plaque rupture occurs, the exposed tissues clot rapidly, leading to vessel obstruction.

Adapted from Libby & Theroux (2005).

water pipe (Libby, 2006). It is now understood that several immune responses are involved in disease progression and plaque forms within the blood vessel tissues rather than in the lumen (Libby, 2002). Acute cardiovascular events usually occur after the rupture of previously formed plaques, resulting in clot formation of thrombus (Libby, 2002).

Atherosclerosis is initiated by the formation of lesion. When endothelial cells of the artery are affected by risk factors, e. g. certain bacterial products, dyslipidemia, vasoconstrictor hormones or proinflammatory cytokines, cell injury is induced, releasing adhesion molecules that attract the blood leukocytes to stick on (Ramos *et al*, article in press). The leukocytes then transmigrate to the subendothelium, as attracted by the chemoattractant cytokines stimulated by the cardiovascular risk factors (Libby & Theroux, 2005). Smooth muscle cells then continue to grow around the plaque as fibrous caps. Dyslipidemia, on the other hand, promotes the invasion of fatty substance-rich LDL and VLDL into the artery, where such lipoproteins are ingested by the leukocytes and become oxidized. This leads to the build up of fatty streak along the artery, followed by calcification and reduced elastic recoil of the vessel (Aikawa & Libby, 2004; Tang *et al*, 2006).

Plaque deposition does not necessarily lead to stenoses (narrowing of blood vessels) and most plaques actually grow outward rather than inward (Arnett *et al*, 1979), although some may progress in sudden spurts rather than continuously during ruptures (Bruschke *et al*, 1989; Yokoya *et al*, 1999). In plaque ruptures, the fatty tissues are released and exposed, promoting clot, leading to thrombus (blood clot), which may result in lumen closure. Closure in coronary artery may rapidly and suddenly reduce blood flow (and hence oxygen supply) to cardiac muscles, causing myocardial infarction.

1.2 Cholesterol homeostasis

1.2.1 Liver as the main organ for cholesterol metabolism

Liver produces over 50% of newly synthesized cholesterol, and is considered the single most important organ for the regulation of cholesterol metabolism in the body (Dietschy *et al*, 1993; Kruit *et al*, 2006; Repa & Mangelsdorf, 2000; Yao *et al*, 2007). It was estimated that there was about 100 grams of cholesterol in a 70-kg man and 600-900 milligrams of cholesterol synthesized daily, whereas a mouse had a cholesterol pool of 50 milligrams and 3 milligrams of cholesterol was synthesized per day (Gylling & Miettinen, 1992; Gylling *et al*, 1994; Schwarz *et al*, 1998; Vuoristo & Miettinen, 1985).

1.2.2 Regulatory sites of cholesterol metabolism

Cholesterol balance is maintained in several regulatory points: intestinal cholesterol absorption, hepatic LDL cholesterol uptake, hepatic HDL-cholesterol uptake, hepatic cholesterol *de novo* synthesis, hepatic excretion into bile acids and cholesterol ester transfer between lipoprotein classes in the blood (Figure 1.3).

1.2.2.1 Regulation of cholesterol absorption by acyl coenzyme A: cholesterol acyltransferase (ACAT)

Cholesterol is uptaken and stored in cells in the form of cholesterol esters and the conversion is catalyzed by ACAT (Buhman *et al*, 2000; Chang *et al*, 1997) (Figure 1.4). Two ACAT genes, designated as ACAT-1 and ACAT-2, have been identified in mammals (Chang *et al*, 2006; Meiner *et al*, 1996). ACAT-1 has been found in macrophages of atherosclerotic lesions, hepatic cells, adrenal glands, neurones and intestinal cells, while ACAT-2 is primarily expressed in the intestinal

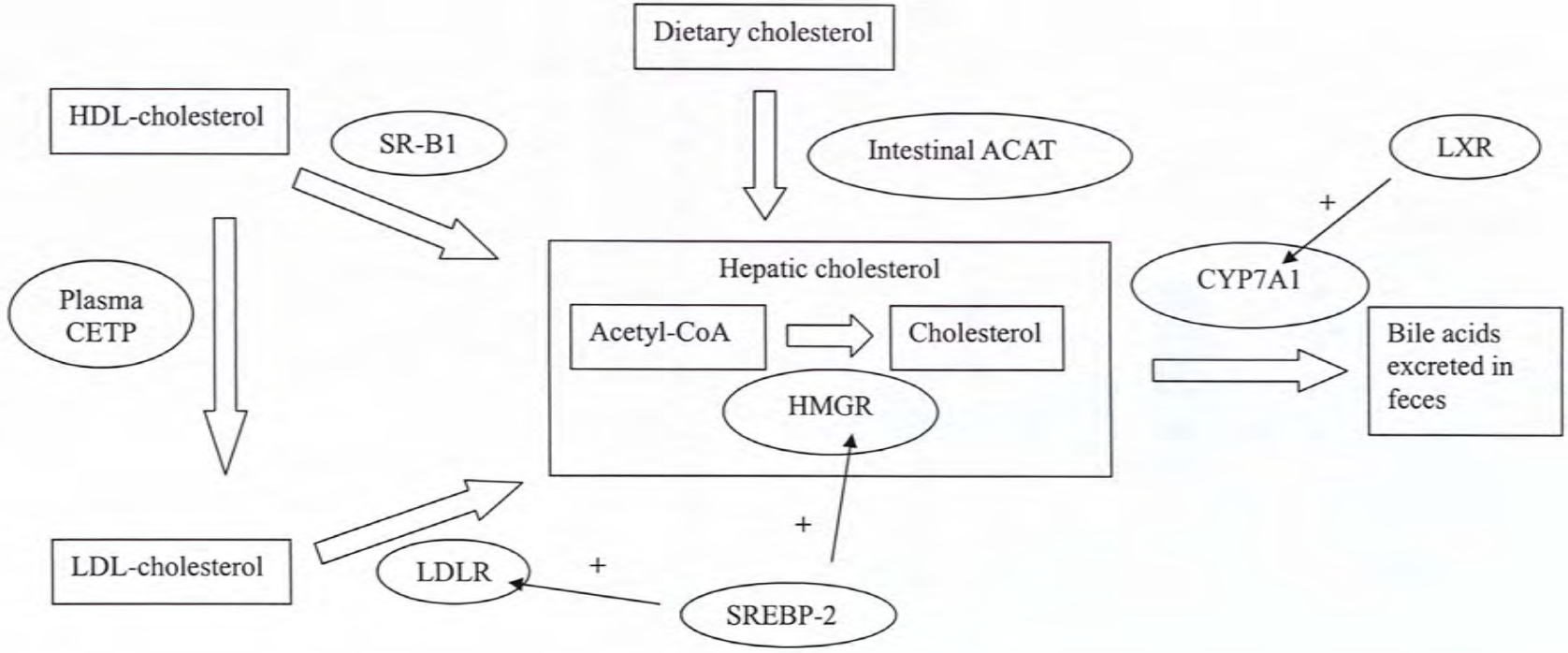

Figure 1.3
Regulation sites of cholesterol metabolism
Cholesterol metabolism is together regulated by intestinal acyl cholesterol: acyltransferase (ACAT), plasma cholesterol ester transfer protein (CETP), hepatic scavenger receptor-B1 (SR-B1), low density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and cholesterol-7α-hydroxylase (CYP7A1), while LDLR and HMGR are upregulated by sterol regulatory element-binding protein 2 (SREBP-2) and CYP7A1 is upregulated by liver-X-receptor (LXR).

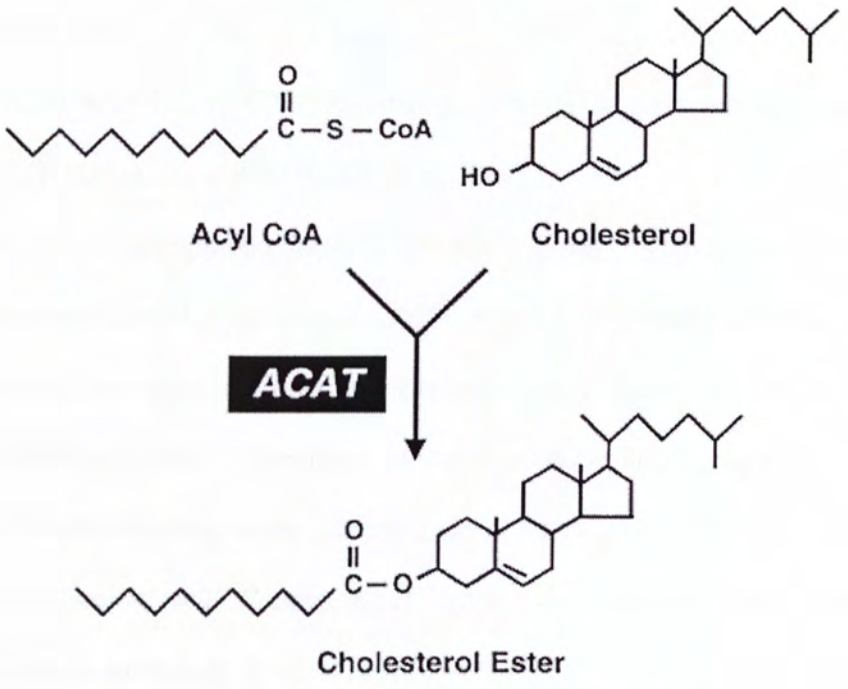

Figure 1.4

Activity of acyl cholesterol: acyl transferase (ACAT)

ACAT catalyzes the esterification between cholesterol and a fatty acid moiety.

Adapted from Chen (2001).

mucosa and the liver (Burnett *et al*, 2005; Cases *et al*, 1998; Chang *et al*, 2000; Jiménez-López *et al*, 2006; Lee *et al*, 1998; Miyazaki *et al*, 1998; Sakashita *et al*, 2000; Sakashita *et al*, 2003). In a quantitative study, ACAT-1 mRNA was found to predominate over that of ACAT-2 in the liver, while ACAT-2 mRNA predominated over ACAT-1 in intestinal cells (Smith *et al*, 2004).

ACAT-2 is an important factor in intestinal cholesterol absorption (Parini *et al*, 2004; Repa *et al*, 2004; Willner *et al*, 2003). ACAT-2 deficiency had been reported to cause reduced intestinal cholesterol absorption in mice (Buhman *et al*, 2000). It has previously been shown by our laboratory that decrease in intestinal ACAT activity simultaneously occurred with increased fecal sterol excretion in hamsters fed conjugated linoleic acid (Yeung *et al*, 2000). Inhibition on ACAT activity by pharmaceutical inhibitors, e. g. NTE-122 and F-12511, has also been reported to associate with reduction on atherosclerosis incidence (Azuma *et al*, 2001; Rival *et al*, 2002).

There has been an increasing number of investigations on the possibility of achieving hypocholesterolemic and anti-atherosclerotic results from pharmaceuticals targeting at ACAT (Alegret *et al*, 2004; Chang *et al*, 2006).

1.2.2.2 Sterol regulatory element-binding protein 2 (SREBP-2) as a transcription factor for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and low-density lipoprotein receptor (LDLR)

SREBP-2 is a protein belonging to the sterol regulatory element-binding protein family, which has been shown to play an important regulatory role in fatty acid and cholesterol synthesis (Eberlé *et al*, 2004).

There are three isoforms of SREBP found, namely SREBP-1a, SREBP-1c and SREBP-2 (Weber *et al*, 2004). SREBP-1a and SREBP-1c are encoded by the same gene, located on the human chromosome 17p11.2 (Hua *et al*, 1995b), while SREBP-2 is encoded by a separate gene, located on 22q13 (Miserez *et al*, 1997). SREBP-1a and SREBP-1c are generated by alternative transcription start sites of the same gene and are responsible for the regulation of fatty acid metabolism (Liang *et al*, 2002), while SREBP-2 is involved in the regulation of cholesterol acquisition (Repa & Mangelsdorf, 2000).

Structurally, SREBPs are basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors, having a molecular size of about 125 kDa constituted by 1150 amino acids (Weber *et al*, 2004). They are membrane-bound proteins found on the endoplasmic reticulum and nuclear envelope (Hua *et al*, 1995a). They become biologically active after activation by proteolytic cleavage (Eberlé *et al*, 2004). Under appropriate conditions, SREBP cleavage-activating protein (SCAP) escort SREBP into the Golgi apparatus, where two proteases, namely site-1-protease (S1P) and site-2-protease (S2P) sequentially cleave the hydrophilic loop of SREBP, releasing the mature (nuclear) form SREBP into the cytosol (Matsuda *et al*, 2001; Sakai *et al*, 1998; Wang *et al*, 1994).

SREBP-2 then binds to the sterol regulatory element on the chromosome, turning

up the expression of a number of cholesterol-generating genes (Sakakura *et al*, 2001), including HMGR and LDLR (Horton *et al*, 1998). A diagram explaining the activation process of SREBPs is shown in Figure 1.5.

1.2.2.3 Roles of LDLR

LDLR is a trans-membrane, cell surface-residing and membrane-bound protein that belongs to the LDL-receptor related protein family (Hussain *et al*, 1999; May *et al*, 2007). Discovered and characterized by Brown and Goldstein (1986), this 839-amino acid glycoprotein is responsible for receptor-mediated uptake and catabolism of LDL-cholesterol from the blood. LDLR is primarily expressed in the liver and adrenal gland (Kim *et al*, 1996).

LDLR primarily recognizes ApoB-100, which is found on the surface of LDL particles (Brown & Goldstein, 1974; Goldstein & Brown, 1974). It also recognizes ApoE, which could be found on the beta-migrating form of VLDL and on certain intermediate and high density lipoproteins (Innerarity & Mahley, 1978; Weisgraber *et al*, 1978). As the LDL particles bind to and complex with LDLR, clarithin-coated pits are formed and the complex enters the cell via endocytosis (Anderson, 1977a; Anderson, 1977b). The vesicles containing the complex are then transported to the endosome, where the LDL is released from LDLR in the low pH environment (Davis *et al*, 1987). LDLR is then recycled back to the cell surface again (Goldstein *et al*, 1985). A schematic diagram on the process of LDL uptake from the blood via LDLR mediated endocytosis is shown in Figure 1.6.

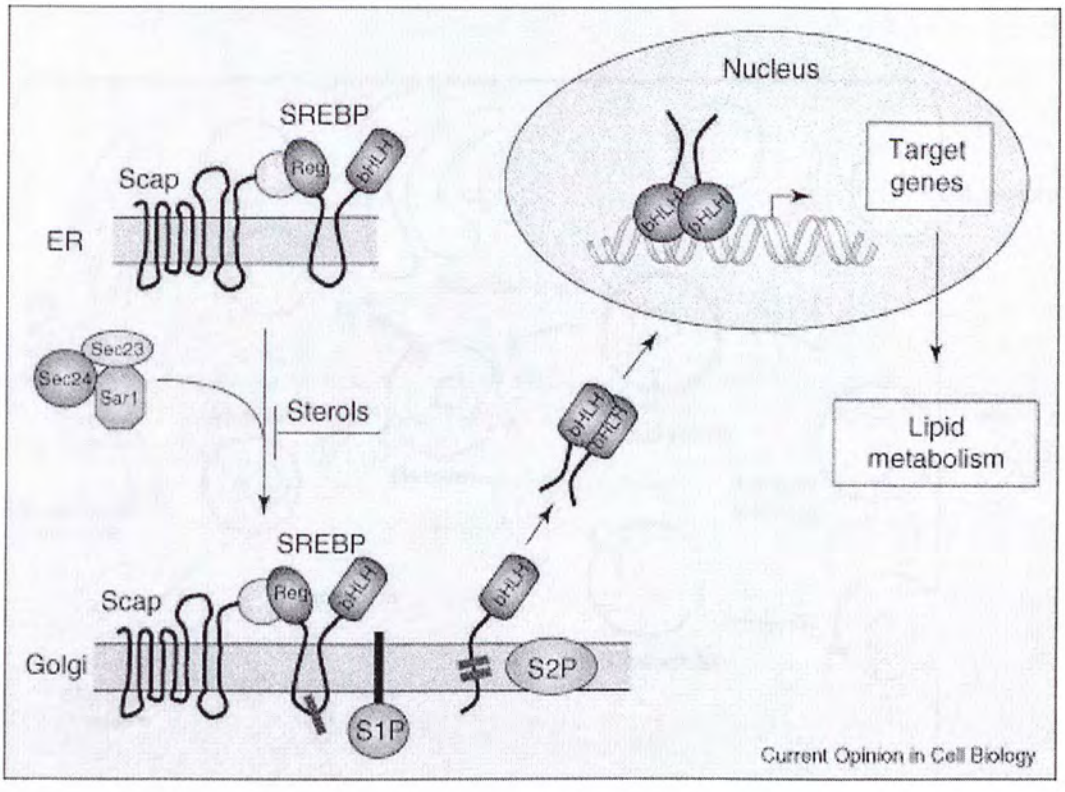

Figure 1.5

Activation of SREBPs through SREBP cleavage-activating protein (SCAP) escort and site-1-protease (SIP) and site-2-protease (S2P) cleavage.

Nuclear SREBPs eventually enter the nuclear where they bind to the sterol regulatory element on the chromosome to activate gene expressions. Adapted from Bengoechea-Alonso & Maria (2007).

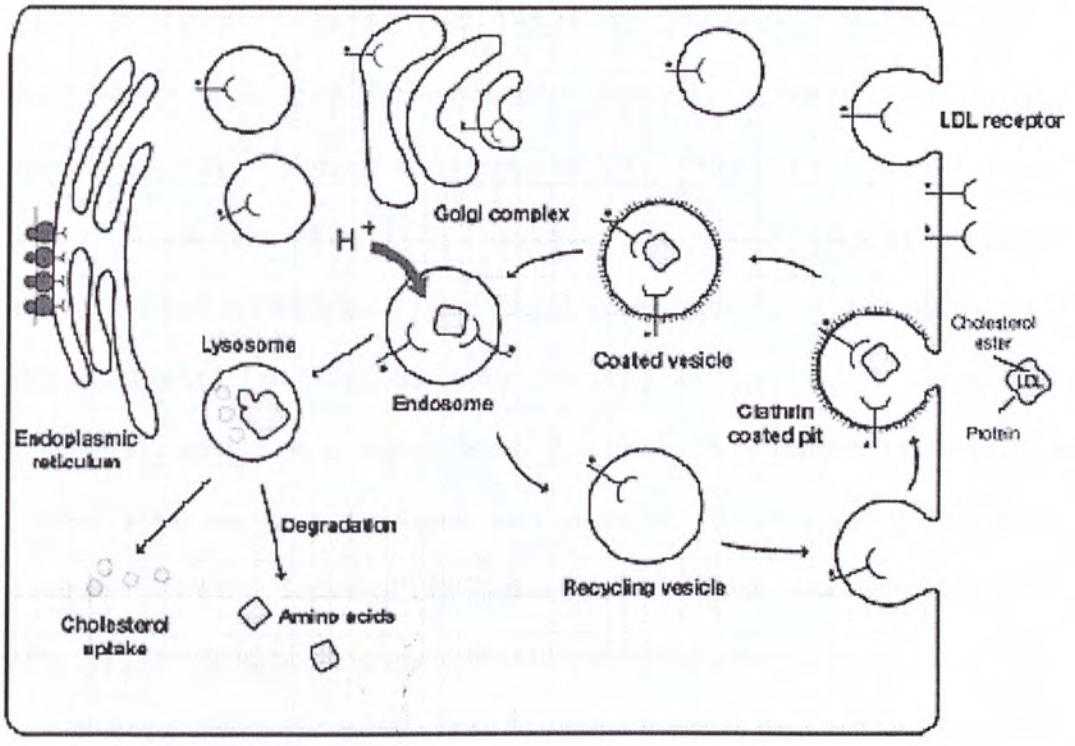

Figure 1.6

Schematic diagram on LDL endocytosis through LDLR

Modified from Jeon *et al* (2005).

Polymorphism or mutation in LDLR has been identified in certain individuals and been attributed to familial hypercholesterolemia, a genetic defect in LDLR in some individuals (Soutar & Naoumova, 2007; Widhalm *et al*, 2007). LDLR-mediated endocytosis is the main route of clearance of LDL cholesterol and cholesterol ester from the blood (Jones *et al*, 2007). The number of functional LDLR on cell surface has been shown to determine the rate of LDL catabolism (Hobbs *et al*, 1992). It has also been reported that when intracellular cholesterol decreases (Goldstein *et al*, 1985) or under the stimulation of certain factors (Liu *et al*, 1997), nuclear form SREBP is released to the nucleus and activates the transcription of LDLR and HMGR (to be discussed in 1.2.2.4). Increased LDLR then promotes uptake of cholesterol from the LDL in blood and intracellular cholesterol balance is restored.

In recent years, modulation of LDLR activity has been proposed as a strategy to reduce blood LDL cholesterol (Issandou, 2006). Increasing the number of LDLR on cell surface has been shown to be effective in promoting removal of LDL cholesterol from the blood and reducing blood LDL cholesterol level (de Medina *et al*, 2004; Defesche, 2004; Rudenko & Deisenhofer, 2003).

1.2.2.4 Rate limiting role of HMGR in cholesterol *de novo* synthesis

Besides dietary intake, cholesterol could also be *de novo* synthesized from acetyl-CoA (Figure 1.7), in which reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) into mevalonate by HMGR is the rate-limiting step along the pathway (Goldstein & Brown, 1990).

Being the rate-limiting enzyme in cholesterol synthsis, HMGR is one of the most highly regulated enzymes in nature (Goldstein & Brown, 1990). Transcription of HMGR is regulated mainly through allosteric feedback regulation by cholesterol and

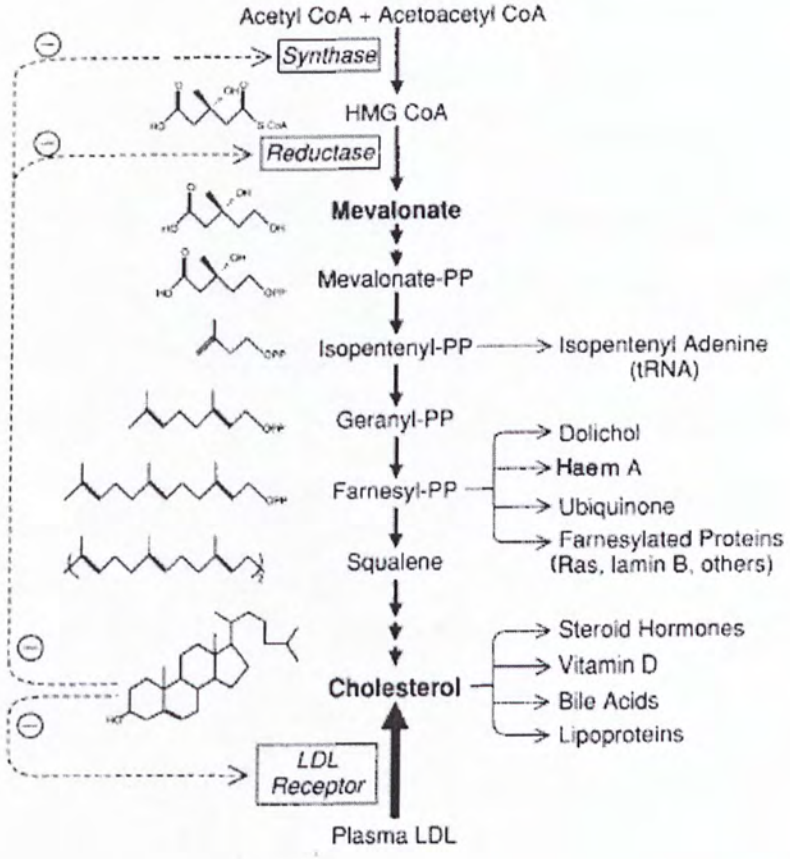

Figure 1.7

The mevalonate pathway of cholesterol de novo synthesis

HMGR acts as the rate-limiting enzyme in the pathway, while cholesterol itself acts as an allosteric inhibitor on HMGR by feedback regulation.

(Adapted from Goldstein and Brown (1990))

by SREBP-2. When intracellular cholesterol depletes, nuclear form SREBP-2 is released by proteolytic enzymes and enters the nucleus, where it activates transcription of HMGR (Horton *et al*, 1998). Increased HMGR promotes cholesterol synthesis to restore intracellular balance (Repa & Mangelsdorf, 2000).

Inhibiting HMGR activity by pharmaceuticals is an established strategy to reduce blood LDL cholesterol, and statins are the first drugs approved for such purpose. Introduced in the U. S. since 1987, lovastatin was the first drug available for clinical use for hypercholesterolemia therapy (Bays, 2006). Statins have a similar structure as HMG-CoA, and hence they bind to the active site of HMGR, sterically inhibiting the binding of HMG-CoA to HMGR (Vaughan & Gotto, 2004). Clinical trials reported that statins prescription reduced both total cholesterol (TC) and LDL-cholesterol concentrations in blood by 20-60% (Igel *et al*, 2002) while decreasing major coronary and major cerebrovascular events by 29% and 14% respectively (Thavendiranathan *et al*, 2006).

1.2.2.5 Roles of liver-X-receptor-α (LXR-α) in cholesterol catabolism

LXR, like SREBP-2, is a member in the nuclear receptor superfamily. There are two conserved subtypes of LXR identified, LXR-α and LXR-β. LXR-α is primarily expressed in the liver, adipose tissue and macrophages while LXR-β is almost ubiquitously expressed (Repa & Mangelsdorf, 2000). LXR-α mainly functions as an activator of the transcription of cholesterol-7α-hydroxylase, a key enzyme in cholesterol catabolism, while LXR-β is reported to promote cholesterol reverse transport from the peripheral to the liver (Lund *et al*, 2006).

LXR-α is a sensor for oxysterols, which are the oxidation products of cholesterol (Janowski *et al*, 1996). LXR-α forms heterodimer with retinoid-X-receptor (RXR),

and under high intracellular oxysterol condition, which in turn implies high intracellular cholesterol, these oxysterols bind to LXR-α and the ligand-bound LXR-α becomes activated (Lehmann *et al*, 1997). Activated LXR-α then binds to its response element, LXRE, in the promoter region of the cholesterol-7α-hydroxylase (CYP7A1) and initiates its transcription (Figure 1.8).

The potential of LXR as target for hypercholesterolemia therapy has been studied extensively in recent years (Bruemmer & Law, 2005; Michael *et al*, 2005). Synthetic and natural LXR agonists (substances that bind to a receptor and activate its response) have been shown to activate LXR-dependent genes transcription and/or promote liver cholesterol efflux (Bramlett *et al*, 2003; Deng *et al*, 2006). Increased activated LXR activates CYP7A1 expression, which in turn increases bile acid synthesis. This promotes cholesterol output from the liver and depletes hepatic cholesterol content. Theoretically, this should stimulate hepatic LDLR activity and the ultimate outcome is increased clearance of LDL cholesterol from the blood, leading to hypocholesterolemic response. However, the data suggest that LXR may also induce fatty acid synthesis, and therefore use of LXR agonists could not be guaranteed to be anti-atherosclerotic at present (Millatt *et al*, 2003).

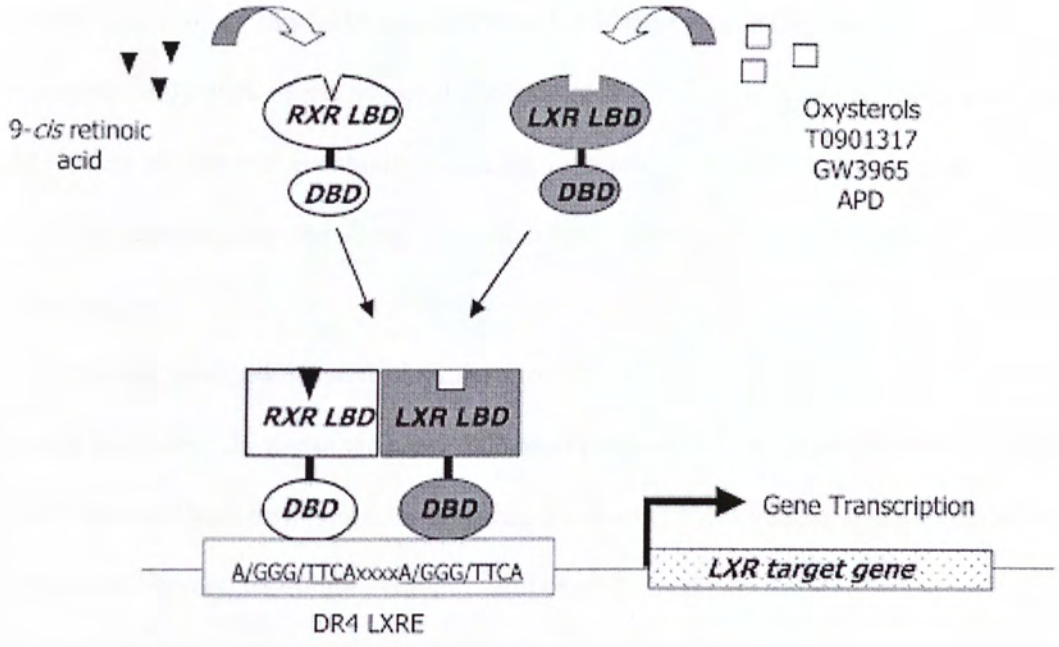

Figure 1. 8

Activation of gene transcription by LXR

Adapted from Millatt (2003).

1.2.2.6 Roles of CYP7A1 in catabolism of cholesterol into bile acids

The liver is the only organ in the body capable of excreting excess cholesterol and its metabolites, thus maintaining cholesterol homeostasis (Vlahcevic *et al*, 1999). Approximately 50% of the excreted cholesterol is eliminated through conversion into bile acids which are eventually excreted in feces, while the remainder is mainly directly excreted into the feces through biliary canaliculi via a mechanism poorly understood.

On one hand, conversion of cholesterol into bile acid excretes excess cholesterol out of the body. On the other hand, bile acid products act as an emulsifier and help lipids in the chyme turn into finer droplets, facilitating the digestion and absorption of fatty acids, sterols, fat-soluble vitamins and other hydrophobic nutrients.

Bile acids may be synthesized via the classical (neutral) or the acidic (alternative) pathways. Human studies revealed that most (70-95%) of bile acids were synthesized via the classical pathway and the acidic pathway was comparatively inefficient (Swell *et al*, 1980).

Cholesterol-7α-hydroxylase is the rate limiting enzyme in bile acid synthesis in the neutral pathway, which involves at least 15 enzymatic reactions in four organelles in the hepatocytes (Vlahcevic *et al*, 1992). Being a member of the cytochrome (CYP) family, cholesterol-7α-hydroxylase is also named CYP7A1. It is responsible for the 7α hydroxylation of cholesterol into 7α-hydroxycholesterol, the first and rate-limiting step in bile acid synthesis (Norlin & Wikvall, 2007; Pikuleva, 2006; Vlahcevic *et al*, 1999). (Figure 1.9)

Studies have shown that altering CYP7A1 activity could result in changes in blood cholesterol level (Hubacek & Bobkova, 2006). Use of a bile acid sequestrant reduces body bile acid pool by 40%, activating CYP7A1 activity and hence increases

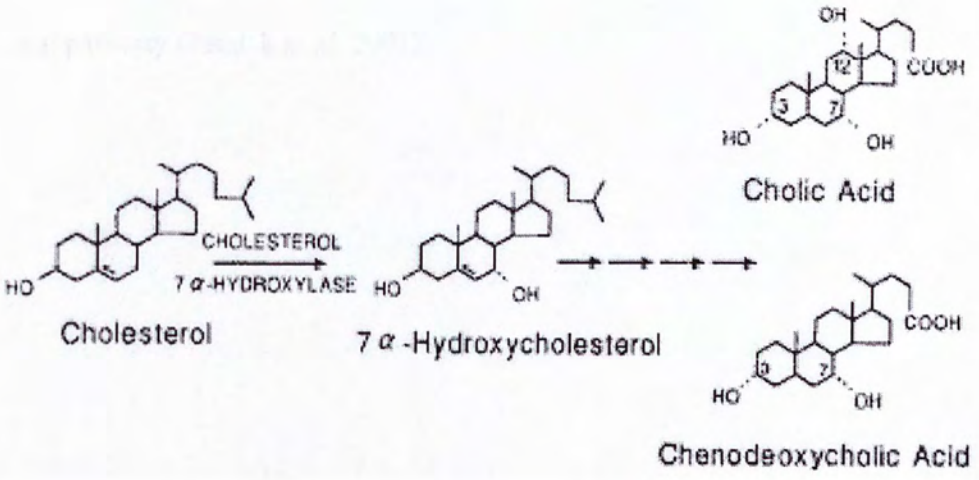

Figure 1.9

Role of cholesterol-7α-hydroxylase in the classical pathway of bile acid synthesis

Modified from Jelinek *et al.*(1990).

bile acid output, and the ultimate result is a reduction of LDL-cholesterol by 15-26% (Insull, 2006). In cultured cells, overexpression of CYP7A1 by molecular techniques in HepG2 and PHH cells significantly increased bile acid synthesis through the classical pathway (Pandak *et al*, 2001).

1.2.2.7 Roles of cholesterol ester transfer protein (CETP) in maintaining cholesterol distribution in blood

CETP is a 476-amino acid glycoprotein that is mainly secreted by the liver and adipose tissue into the circulating blood. Bound mainly to HDL, CETP catalyzes the transfer of cholesterol ester from HDL to apoB-containing lipoproteins (e.g. LDL, VLDL, VLDL remnants) and that of triglyceride from apoB-containing lipoproteins to HDL in the blood (Le Goff *et al.*, 2004). Since HDL mainly transports cholesterol from the peripheral to the liver for catabolism via scavenger receptor route in reverse cholesterol transport (RCT), reduction in HDL-cholesterol by CETP decreases direct RCT (Shah, 2007) and may increase the risk of atherosclerosis.

Besides lipid transfer, CETP also modifies the size of circulating lipoproteins into small, dense ones (Chung *et al.*, 1998). Present epidemiological data suggest that reduced LDL size is associated with a higher risk of CHD (Gardner *et al.*, 1996; Lamarche *et al.*, 1997; Stampfer *et al.*, 1996). These further suggest that CETP activity is pro-atherosclerotic.

However, there are also opinions that CETP carries anti-atherosclerotic property through its enhancement in indirect RCT. Redistribution of cholesterol ester from HDL into LDL allows transfer of cholesterol ester to the liver via LDLR (Figure 1.10). In species having a high expression level of CETP like rabbit, this route contributes about 70% of cholesterol uptake from HDL (Barter *et al.*, 2003; Goldberg *et al.*, 1991).

However, in human, the reduction of direct RCT by CETP seems not be neutralized by its increase in indirect RCT. Patients having mutation in CETP genes were found to have higher HDL-cholesterol levels than normal subjects (Inazu *et al.*, 1990). A recent human trial found out that CETP inhibition by Torcetrapib, a CETP

inhibitor, increased HDL-cholesterol by 60% while decreasing LDL-cholesterol by 20%, yet not affecting atherosclerosis progression (Nissen *et al.*, 2007). Ansell and Hobbs (2006) reviewed 30 reports about vaccine and pharmacological CETP inhibition and concluded that pharmacological CETP inhibitors generated a more consistent increase in HDL-cholesterol. Nonetheless, at least seven classes of CETP inhibitors are now under development and CETP inhibition is now a new target in the treatment of dyslipidemia (Shah, 2007).

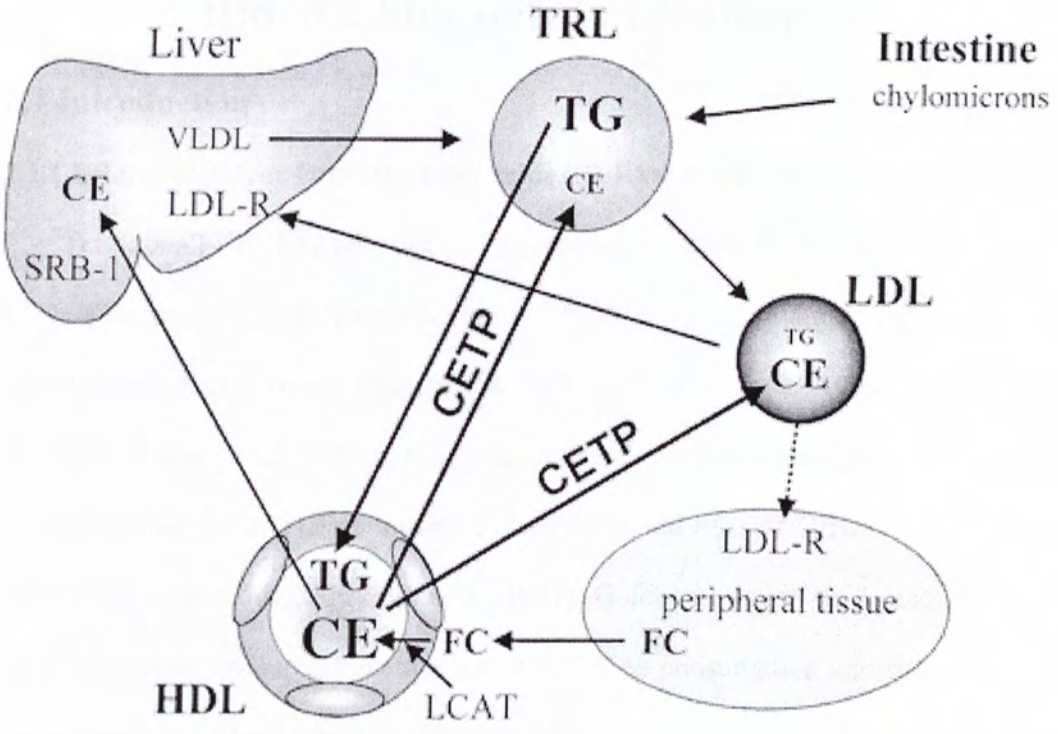

Figure 1.10

Roles of CETP in redistributing lipids in the blood. Key: TRL: triglyceride-rich lipoprotein

(Adapted from Barter (2003))

Chapter 2
Effect of Octadecaenoic Acids on Blood Cholesterol in Hamsters

2.1 Introduction

2.1.1 Effects of polyunsaturated fatty acids (PUFAs) on blood cholesterol

Traditionally, PUFAs are regarded as protective factors for CHD (Grundy *et al*, 1982; Krauss *et al*, 2000; Kris-Etherton *et al*, 2002), while saturated fatty acids are regarded as inducing factors (Boniface & Tefft, 2002; Esrey *et al*, 1996; Goldbourt *et al*, 1993; Kushi *et al*, 1985). However, human epidemiological studies reported inconsistent effects of PUFAs on blood cholesterol and risks of CHD. Mensink *et al* (2003), Oh *et al* (2005), Shekelle *et al* (1981), Goldbourt *et al* (1993) and Hu *et al* (1997) found an inverse association between PUFAs consumption and risk of CHD, while some others found no association (Ascherio *et al*, 1996; Garcia-Palmieri *et al*, 1980; Gordon *et al*, 1981; Kromhout & de Lezenne, 1984; Morris *et al*, 1977).

Investigations in individual PUFAs, however, generated more consistent results. Longer-chain ω-3 PUFAs from fish oil, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), reduced CHD risk (de Lorgeril *et al*, 1999; Lichtenstein, 2006). These data suggest that the specific effects of these individual fatty acids on the cardiovascular system deserve more investigation.

2.1.2 Differential effects of 18-C PUFAs on lowering blood cholesterol *in vivo*

The two essential 18-C PUFAs, namely linoleic acid (LA) and α-linolenic acid (LN), are the parent compounds of other ω-6 and ω-3 PUFAs respectively. In recent years, the conjugated forms of these fatty acids have also received scientific attention (Fischer-Posovszky *et al*, 2007; Koba *et al*, 2007; Ledoux *et al*, 2007; Purushotham *et*

al, 2007; Ribot *et al*, 2007). It had been shown in our lab that both LA and conjugated linoleic acid (CLA) decreased blood cholesterol in hamsters, but CLA reduced intestinal ACAT activity while LA did not (Yeung *et al*, 2000). In contrast, LN but not conjugated linolenic acid (CLN) was hypocholesterolemic in hamsters (Yang *et al*, 2005).

2.1.3 Structures, metabolism and conjugation of octadecaenoic acids (ODA)

LA and CLA are 18-C PUFAs of similar structures that cannot be synthesized in humans. Both LA and CLA can be desaturated and elongated into longer chain PUFAs. LA can be converted into arachidonic acid by Δ-6 desaturase and elongase while LN can be converted into EPA and DHA by Δ-3 desaturase and elongase.

The two double bonds in LA are unconjugated while CLA is a group of positional and geometric isomers with the two double bonds being in conjugation. LN is a more highly unsaturated form of 18-C fatty acid and has three unconjugated double bonds, while CLN, a group of isomers of LN, have three conjugated double bonds. The structures of LA, CLA, LN and CLN are shown in Figure 2.1.

2.1.4 Objectives

Previous studies demonstrated that four structurally similar 18-C PUFAs, namely LA, CLA, LN and CLN affected blood cholesterol and cholesterol metabolism differently (Yang *et al*, 2005; Yeung *et al*, 2000). However, the underlying mechanism is not established. The present study consisted of two experiements. Experiment 1 was conducted to investigate the effect of LA, CLA, LN and CLN on blood cholesterol and five cholesterol-regulating proteins, namely, SREBP-2, HMGR, LDLR, LXR and CYP7A1 in hamsters. Experiment 2 was conducted to investigate the effect of LA, CLA, LN and CLN on intestinal cholesterol absorption and ACAT activity.

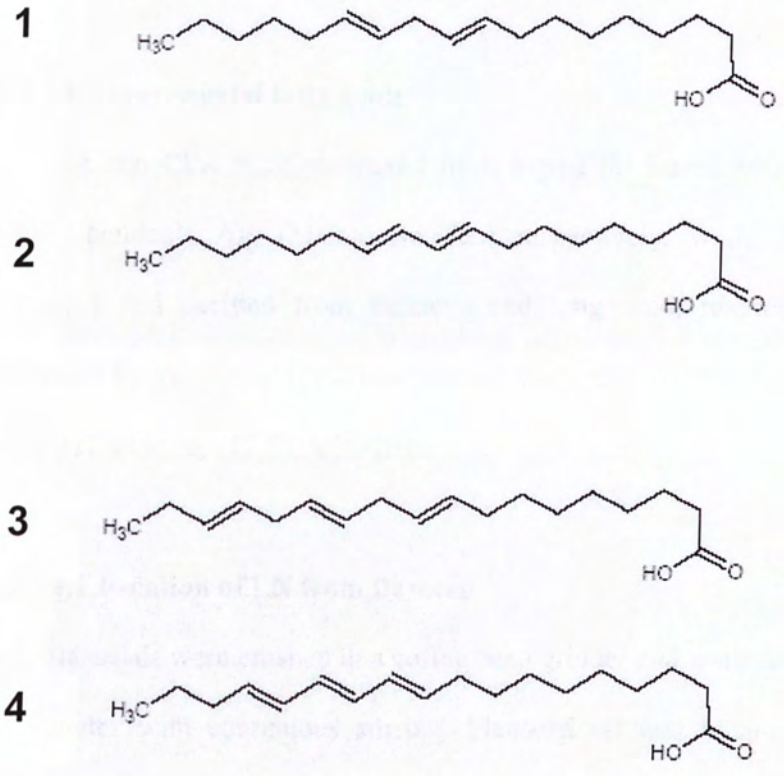

Figure 2.1

Schematic diagram showing the simplified structures of: 1, linoleic acid (LA); 2, conjugated linoleic acid (CLA); 3, linolenic acid (LN); 4, conjugated linolenic acid (CLN).

2.2 Experiment 1

2.2.1 Materials and methods

2.2.1.1 Experimental fatty acids

LA and CLA were purchased from Sigma (St Louis, MO, USA) and Larodan Fine Chemicals AB (Malmö, Sweden) respectively, while LN and CLN were extracted and purified from flaxseed and tung seed, respectively, as previously described by Yang *et al* (2005) with slight modifications. The CLA was a 1:1 mixture of c9, t11 and t10, c12 CLA isomers.

2.2.1.1.1 Isolation of LN from flaxseed

Flaxseeds were crushed in a coffee bean grinder and were soaked in n-hexane for 90 minutes with continuous stirring. Flaxseed oil was obtained after removal of hexane in a rotary evaporator. 130 g of the oil was saponified in 2 L 0.3 M KOH in methanol at 90°C under a gentle nitrogen stream for 2 hours. After acidification of 1 M H_2SO_4 to pH 1.0, LN in the crude fatty acid mixture was isolated by a three-step crystallization process. The mixture was dissolved in three volumes of ethanol and placed in 4°C for 24 hours and the crystals were removed by suction filtration. The process was repeated at -20°C and -50°C in a fatty acids-ethanol ratio of 1:10 (v/v). The final purity of LN was about 80 %.

2.2.1.1.2 Isolation of CLN from tung seed

Tung seeds were ground and soaked in n-hexane. Tung oil was saponified similarly as flaxseed oil in 2.2.1.1.1. The crude fatty acid mixture was placed in 4°C for 8 hours and the CLN-containing yellowish needle-shaped crystals were filtered

and collected. Purity of CLN in these crystals was about 80%.

2.2.1.2 Animals

Male adult Golden Syrian hamsters (*Mesocricetus auratus*, n=50, 113±6g) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. They were randomly divided into five groups (n=10) and housed (2 per cage) in wire-bottomed cages at 23°C in a 12-hour light-dark cycle.

Before the experiment, all the animals were allowed to stabilize by being fed a high fat high cholesterol diet (control diet). From week 0 to week 6, one group were continued to feed on the control diet and the other four groups were fed a similar diet except that they were respectively supplemented with 2% of LA, CLA, LN and CLN. During the study, food was given daily and any uneaten food was discarded. The amount of food consumed was measured each day. The animals had free access to food and distilled water and were weighed weekly. Feces of the animals were saved and collected every week.

Blood (1 mL) was bled from the retro-orbital sinus into a heparinized capillary tube at the end of weeks 0, 3 and 6 after food deprivation for 16 hours. The blood was centrifuged at 3000rpm for 10 minutes and the plasma was collected and stored at -20 °C until analysis.

After the last blood sample collection at the end of week 6, the hamsters were kept for three more days to allow for recovery. They were then sacrificed by nitrogen suffocation with their food available (full stomach) at midnight. Blood was collected from abdominal artery into a vacuum heparinized tube, centrifuged at 800 × g at 10 minutes and the plasma was collected and stored at -20°C until analysis. The liver, heart, kidney, adipose tissue (perirenal and epididymal pads) were removed, washed

in saline, weighed, frozen in liquid nitrogen and stored at -80°C until analysis.

2.2.1.3 Diets

The control diet was prepared by mixing the following ingredients in proportion (g/kg diet): cornstarch, 488; casein, 242; lard, 100; sucrose, 119; mineral mix AIN-76, 40; viamin mix AIN-76A, 20; DL-methionine, 1; cholesterol, 1. The four experimental diets were prepared by adding 2% by weight of the fatty acids into the control diet respectively. The powered diets were mixed with a gelatin solution (20g/L) in a ratio of 200g diet per liter of solition (Table 1). Once the gelatin had set, the diets were cut into pieces of approximately 10 g cubes and stored frozen at -20°C.

2.2.1.4 Plasma lipid measurements

Plasma total cholesterol (TC) and triglyceride (TG) were measured by commercial enzymatic kits from Infinity (Waltham, MA, U. S. A.) and Stanbio Laboratories (Boerne, TX, U. S. A.) respectively according to the manufacturer's instructions. For measurement of plasma HDL-cholesterol, LDL and VLDL were first precipitated with phosphotungstic acid and magnesium chloride using a commerical kit (Stanbio) and HDL-cholesterol in the supernatant was determined similarly as did total cholesterol. Non-HDL cholesterol was calculated by deducting HDL-cholesterol from TC.

2.2.1.5 Plasma CETP activity measurement

Plasma CETP activity of the hamsters was measured by a commercial kit (Amersham Pharmacia Biosciences, Piscataway, NJ, U. S. A.) according to the manufacturer's instructions with slight modifications. In brief, 10μl of ^{3}H-HDL, biotinylated LDL and assay buffer were mixed with 5μl of hamster plasma samples and the mixture was incubated at 37°C for 16 hours. After that, a stop solution

containing SPA-beads that specifically bound to biotinylated LDL was added to detect [^{3}H] radiation emitted from LDL. The reaction mixture was read on a Beckman LS6500 scintillation counter (Beckman, Fullerton, CA, U. S. A.).

Table 1 Compositions for the diets of the five groups of hamsters (g/ kg diet)

	Control	LA	CLA	LN	CLN
Cornstarch	488	478	478	478	478
Casein	200	196	196	196	196
Sucrose	150	147	147	147	147
Mineral mix (AIN-76)	40	39	39	39	39
Vitamin mix (AIN-76A)	20	20	20	20	20
d-methionine	1	1	1	1	1
Cholesterol	1	1	1	1	1
Lard	100	98	98	98	98
LA	0	**20**	0	0	0
CLA	0	0	**20**	0	0
LN	0	0	0	**20**	0
CLN	0	0	0	0	**20**
Gelatin	20	20	20	20	20

Table 2 Fatty acid composition of the diets for the five groups of hamsters (g/ kg diet)

	Control	LA	CLA	LN	CLN
14:0	2.7	2.6	2.6	2.3	3.5
16:0	16.5	15.2	17.3	18.6	18.1
16:1 n-7	1.9	3.0	1.9	1.7	3.3
18:0	4.9	12.7	9.2	7.7	7.4
18:1 n-9	24.7	24.5	24.9	22.5	22.6
18:2 n-6	12.5	**29.5**	12.1	13.4	14.7
total CLA	0.0	0.0	**16.7**	0.0	0.0
(c9,t11/ t9,c11)-CLA	0.0	0.0	8.6	0.0	0.0
(c10,t12/ t10,c12)-CLA	0.0	0.0	8.1	0.0	0.0
18:3 n-3 (LN)	0.4	0.5	0.8	**17.1**	0.9
total CLN	0.0	0.0	0.0	0.0	**16.8**
(c9,t11,c13)-CLN	0.0	0.0	0.0	0.0	0.5
(c9,t11,t13)-CLN	0.0	0.0	0.0	0.0	13.5
(t9,t11,c13)-CLN	0.0	0.0	0.0	0.0	0.9
(t9,t11,t13)-CLN	0.0	0.0	0.0	0.0	1.9
others	5.2	5.8	4.0	5.2	4.8

2.2.1.6 Measurement of liver SREBP-2, LDLR, HMGR and CYP7A1 protein abundance by Western blotting

Hepatic protein was extracted according to the method described by Vaziri *et al* (1996) with some modifications. In brief, frozen liver was ground in a homogenizing buffer containing 20mM Tris-HCl (pH 7.5), 2mM $MgCl_2$, 0.2M sucrose and Complete® protease inhibitor cocktail (Roche, Mannheim, Germany). The extract was centrifuged at 12,000 *g* for 15 minutes at 4 °C and the supernatant was collected and considered as the 'total protein'. A portion of the total protein was then centrifuged at 35,000 rpm for 60 minutes at 4 °C. The supernatant was removed and the pellet was resuspended in the homogenizing buffer described above and considered as 'membrane protein'. Protein concentration of two fractions was determined using a protein concentration assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U. S. A.).

For the measurement of LDLR, CYP7A1 and HMGR, 100μg of the membrane protein was size-fractionated by 7 % SDS-PAGE at 120V for two hours. The proteins were then transferred to a Hybond-P PVDF membrane (Amersham Pharmacia Biosciences). The membrane was incubated for one hour in blocking solution (5% nonfat milk in 1X TBST) at 4 °C and then overnight in the same solution containing 1:300 anti-LDL receptor antibody (Santa Cruz Biotechnology, Inc., California, U. S. A.) or 1:500 anti-HMGR (Upstate USA Inc., Lake Placid, NY, USA) and 1:200 anti-CYP7A1 (Santa Cruz Biotechnology, California, U. S. A.) whichever appropriate. The membrane was then washed three times for 15 minutes in 1 × TBS and 0.1% Tween-20 and was then incubated for one hour at 4 °C in diluted (1:3000) horseradish peroxidase-linked rabbit anti-goat IgG (Zymed Laboratories Inc., San Francisco, CA, U. S. A.) or donkey anti-rabbit IgG (Santa Cruz Biotechnology). The washes were repeated before the membranes were developed with ECL enhanced

chemiluminescence agent (Amersham Life Science) and subjected to autoradiography for one to five minutes on Super*RX* medical X-ray film (Fuji, Tokyo , Japan). Densitometry was quantified using the computer software Photoshop® (Adobe Systems Inc, CA, U. S. A).

For SREBP-2, 100μg of the membrane protein and 50μg of the total protein aliquots were mixed and simultaneously size-fractionated on 7% SDS-PAGE at 120V for two hours. The remaining procedures were the same as described above except 1:300 anti-SREBP-2 antibody (Santa Cruz Biotechnology) was used as primary antibody.

2.2.1.7 Measurement of hepatic SREBP-2, LDLR, HMGR, LXR, CYP7A1, CETP, SR-B1 and LCAT mRNA by real time PCR

2.2.1.7.1 Extraction of mRNA

Hepatic mRNA was extracted and isolated using the commercial extraction agent Trizol® (Invitrogen, Carslbad, CA, U. S. A.) according to the manufacturer's instructions. In brief, 3 mL of Trizol was added into approximately 300 mg of liver sample and the sample was homogenized with a tissue tearor (Biospec, OK, U. S. A.) on ice. The homogenate was then centrifuged at 4000 × g for 25 minutes at 4 °C. The supernatant was then transferred to a new vial and allowed to stand at temperature for 5 minutes. Chloroform was added to the vial in a homogenate: chloroform ratio of 5:1 and the vial was then vortexed for 15 seconds. After a 3-minute standing at room temperature, the vial was centrifuged at 11900 × g for 15 minutes at 4 °C. The RNA-containing upper aqueous layer was transferred to a new vial and isopropyl alcohol was added in a homogenate: isopropyl alcohol ratio of 2:1. After gently mixing 3 times, the content was allowed to stand for 10 minutes at room temperature

and then centrifuged at 11900 × g for 10 minutes at 4 °C. The supernatant was discarded and the RNA pellet was resuspended with 1 mL of 75% (v/v) ethanol. After vortexing and centrifugation at 7500 × g for 5 minutes at 4 °C, the ethanol was removed and the RNA pellet was air dried. After dissolution in 30 μL DEPC-treated water (0.1% (v/v) of diethyl pyrocarbonate (DEPC) solution in distilled water stirred overnight at room temperature and then autoclaved), the samples were stored at -80°C until analysis.

2.2.1.7.2 Complementary DNA synthesis

Total RNA was converted to complementary DNA (cDNA) by reverse transcriptase (Applied Biosystems, Foster City, CA, U. S. A.). 4μg total RNA was mixed with 1 μl of 50 μM oligo (dT) 25, 1 μl of 3 μg/μl random primers, 2 μl of 10 mM dNTP mixture (all are purchased from Applied Biosystems) and the volume was adjusted to 12 μl with DEPC-treated water. The mixture was incubated at 65°C for 5 minutes and then chilled on ice. 4 μl of 5X M-MuLV reverse transcriptase reaction buffer, 1 μl of 50mM MgCl2, 1 μl M-MuLV reverse transcriptase RNase H minus (250 U/μl) (all are from Applied Biosystems) and 2 μl DEPC-treated water were added. Reverse transcription was carried out in thermocycler (Gene Amp ® PCR system 9700, Applied Biosystems), with programme set as initiation for 10 minutes at 25°C, followed by 50°C incubation for 90 minutes and 85°C for another 5 minutes. The cDNA synthesized was collected and stored at -20°C.

2.2.1.7.3 Real-time polymerase chain reaction (PCR) anaylsis

Real-time PCR analysis was carried out on a Fast Real-time PCR System 7500 (Applied Biosystems) installed with the Sequence Detection Software version

1.3.1.21 (Applied Biosystems). Five genes studied were: SREBP-2 (GenBank accession number U12330), LDLR (GenBank accession number M13877), HMGR (GenBank accession number X00494), LXR (GenBank accession number NM_005693), CYP7A1 (GenBank accession number L04690) and CETP (GenBank accession number M63992). The expressions of target genes were normalized with that of GAPDH (GenBank accession number DQ403055), a housekeeping gene being used as an internal control. The primers and probes were manufactured by Applied Biosystems.

In a PCR run, a 20 µl universal master mixture (Applied Biosystems), containing 10 µl of 2X TaqMan® Fast Universal PCR Master Mix, 2 µl diluted cDNA, 1 µl of 20× Assays-on-Demand Gene Expression Products (consisting of forward and reverse PCR primers, and 1 FAM dye-labeled TaqMan MGB probe) and 7 µl autoclaved distilled water, was first prepared on ice. The reaction mixture was then subject to thermal cycling conditioned follows: heating up from room temperature to 95 °C in 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Fluorescence measurements were recorded during each annealing step. At the end of each PCR run, the data were analyzed by the Sequence Detection Software version 1.3.1.21 and amplification plots were obtained.

Threshold cycle (Ct) values were exported into a Microsoft Excel worksheet for the calculation of gene expression according to the ΔΔCt method (Applied Biosystems). Levels of gene expressions in the treatment groups were presented as a ratio of treatment: control group.

2.2.1.8 Determination of cholesterol in liver

Cholesterol content in the liver were determined by a method described by Chan *et al* (1999). Briefly, 1 mg of stigmastanol (as internal standard) was added into

about 300 mg of liver sample. 15 mL of methanol-chloroform mixture (2:1, v/v) were used to extract lipids from the tissue together with 5 mL of saline, and the chloroform-methanol phase was saved and evaporated to dryness under a nitrogen stream. The lipids were then mildly saponified with 6 mL of m1M NaOH in 90% ethanol at 90°C for 1 hour, followed by the extraction of non-saponified substances (including cholesterol) by 6mL of cyclohexane together with 1 mL of water. The extracted substances were then converted into their trimethylsilyl-ether derivatives by a commercial trimethylsilyl reagent (dry pyridine-hexamethyldisilazane, 9: 3: 1, v/v/v, Sil-A Reagent, Sigma) at 60°C for 1 hour. After flushing with nitrogen to dryness, the cholesterol trimethylsilyl-derivatives resuspended in 500 μL of n-hexane and transferred into GC-vials and were analyzed in a fused silica capillary column (SAC™-5, 30m x 0.25mm internal diameter, Supelco, Inc., Bellefonte, PA, USA) in a Shimadzu GC-14B GLC equipped with a flame ionization detector (Shimadzu, Tokyo, Japan). The column temperature was set at 285°C and held for 25 minutes. Helium was used as the carrier gas at a head pressure of 150 kPa. Amount of cholesterol in the tissue was calculated as a ratio of internal standard added.

2.2.1.9 Determination of fecal neutral and acidic sterols

Neutral and acidic sterols in the feces were determined by a method described by Czubayko (1991) with slight modification. The feces collected were first isolated and dried in lyophilizer, weighed and ground into powder by a coffee bean mill. Stigmasterol (0.5 mg, Sigma) in 200 μL of chloroform was added into a methylation tube as an internal standard for neutral sterols and dried down by a gentle stream of nitrogen. About 300 mg of lyophilized fecal sample was weighed into the methylation tube together with 0.5 mg hyodeoxycholic acid in 200 mL 1N NaOH in 90% EtOH

being added as an internal standard for acidic sterols. The samples were then mildly hydrolyzed in 8 mL 1N NaOH in 90% EtOH at 90°C for 1 hour. Then, 8 mL of cyclohexane together with 1 mL of water were added to extract of total neutral sterols. The methylation tubes were centrifuged to separate the neutral sterol-containing upper cyclohexane phase and the acidic sterol-containing lower aqueous phase. The two phases were separately analyzed as described as below.

2.2.1.9.1 Determination of fecal neutral sterols

The cyclohexane phase was transferred into a new methylation tube and evaporated to dryness under a gentle nitrogen stream. The sterols were converted into their trimethylsilyl derivatives using a commercial trimethylsilyl reagent (dry pyridine-hexamethyldisilazane, 9: 3: 1, v/v/v, Sil-A Reagent, Sigma) at 60°C for 1 hour. After drying by a nitrogen stream, the sterol derivatives were dissolved in 400μL of n-hexane and transferred into a GC vial and read in a fused silica capillary column (SAC™-5, 30 m x 0.25 mm internal diameter, Supelco, Bellefonte, PA, U. S. A.) in a Shimadzu GC-14B GC equipped with a flame-ionization detector (Shimadzu, Kyoto, Japan). Helium was used as the carrier gas at head pressure 150 kPa and the programme was maintained at 285°C for 30 minutes. Each neutral sterol (coprostanol, coprostanone, cholesterol and dihydrocholesterol) was identified by comparing their retention time with that of authentic standards (Sigma). A typical chromatogram is shown in Figure 2.2.

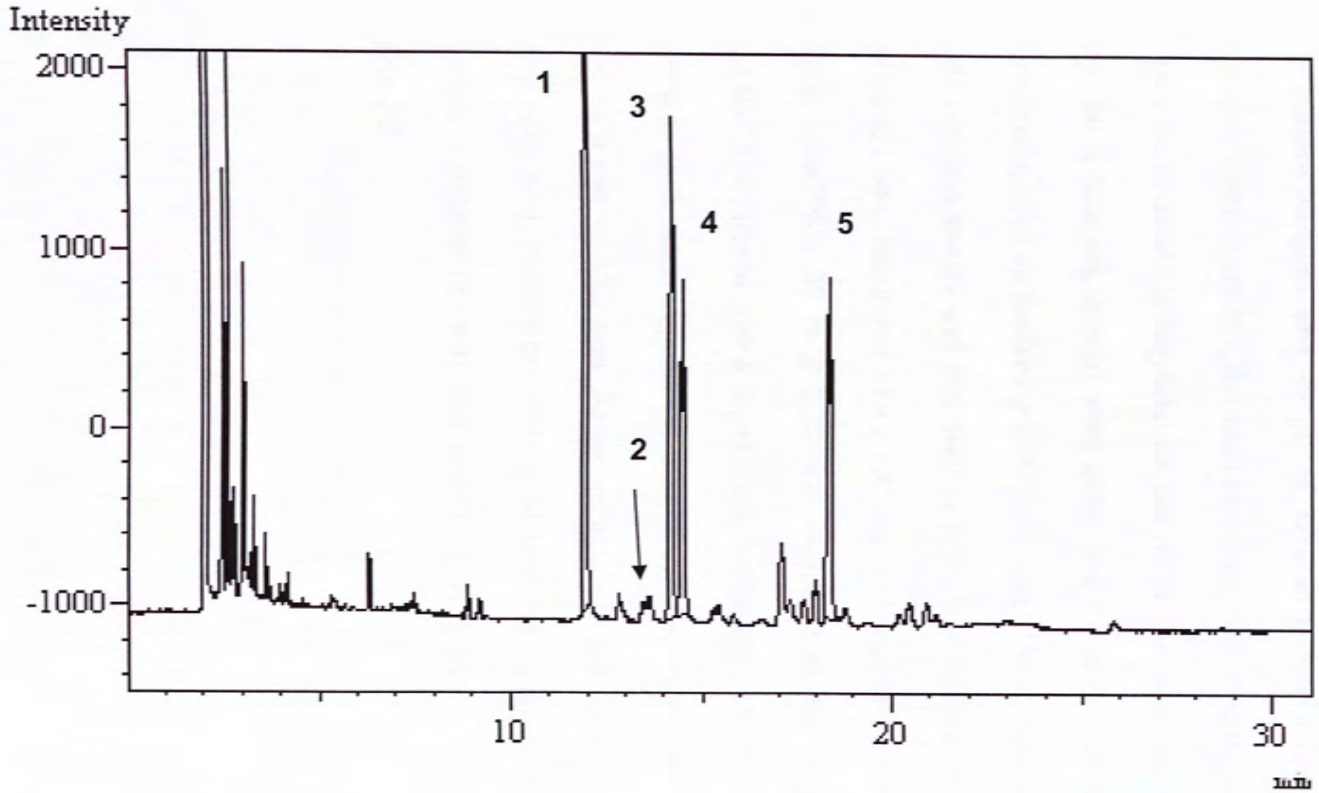

Figure 2.2 A typical gas-chromatograph of fecal neutral sterol analysis. Identification of peaks: 1, coprostanol; 2, coprostanone; 3, cholesterol; 4, dihydrocholesterol; 5, stigmasterol (internal standard).

2.2.1.9.2 Determination of fecal acidic sterols

The bottom aqueous layer described in 2.2.8 was saved for determination of acidic sterol content. 1 mL of 10 N NaOH was added and the mixture was heated at 120°C for 3 hours. 3 mL of water and 1 mL of 25% HCl were then added and the acidic sterols were extracted twice using 7 mL of diethyl ether. The ether phases were pooled and dried under a gentle stream of nitrogen. Then, 2 mL of methanol, 2 mL of 2,2-dimethoxypropane and 40 μL of 37% HCl were added and the mixture was allowed to stand overnight, followed by evaporation to dryness under nitrogen. 300 μL of a commercial trimethylsilyl reagent (dry pyridine-hexamethyldisilazane, 9: 3: 1, v/v/v, Sil-A Reagent, Sigma) were added and the acidic sterols were converted into their trimethylsilyl derivatives at 60°C for 1 hour. The solvent was dried down under a gentle nitrogen stream and dissolved in 300 μL of hexane. After centrifugation, the supernatant was transferred into a GC vial and analyzed on a fused silica capillary column (SAC™-5, 30 m x 0.25 mm internal diameter, Supelco) in a Shimadzu GC-14B GC equipped with a flame-ionization detector (Shimadzu). Helium was used as the carrier gas at head pressure 150 kPa and the programme was set from 230°C to 280°C at a rate of 1°C/ min. Retention time of each acidic sterol (lithocholic acid, deoxycholic acid, chenodeoxycholic acid and cholic acid) was compared with that of authentic standards (Sigma) and identified. A typical chromatogram is shown in Figure 2.3.

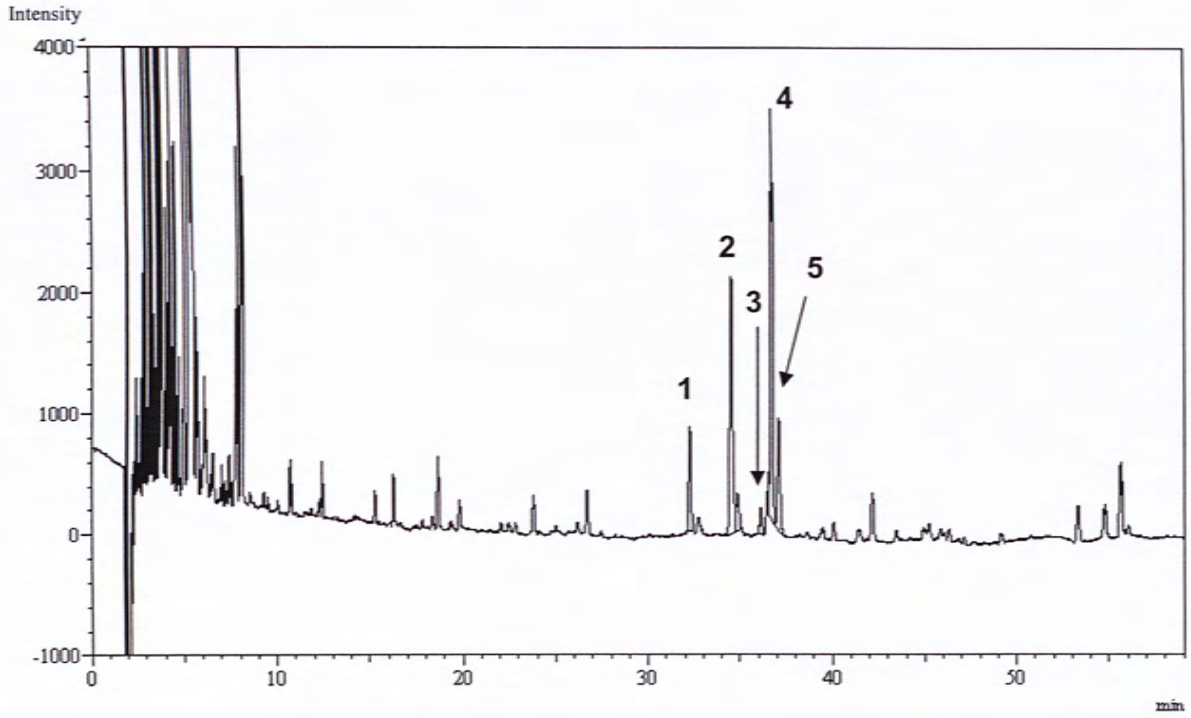

Figure 2.3 A typical gas-chromatograph of fecal acidic sterol analysis. Identification of peaks: 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxycholic acid; 4, cholic acid; 5, hyodeoxycholic acid (internal standard).

2.2.1.10 Statistics

Results were presented as means ± standard deviation (S.D.). Where applicable, statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test, or Pearson's correlation coefficient, using Prism® (Graphpad software, Inc., CA, U. S. A.). Differences between groups were considered significant when $P<0.05$.

2.2.2Results

2.2.2.1 Growth and food intake

The body weight and food intake are shown in Table 2.3. No significant difference in amount of food intake or body weight was observed in the control, LA, CLA, LN or CLN hamsters.

2.2.2.2 Organ weights

Weights of liver, adipose tissues (epididymal and perirenal pads), kidneys and hearts of the hamsters are shown in Table 2.4. Weights of the liver in groups fed CLA and CLN were significantly higher than those of the hamsters fed the control diet. Weights of epididymal and perirenal adipose pads of hamsters fed CLA were significantly lower than those fed the control diet. Kidneys of hamsters fed LN weighed significantly higher than those fed the control diet. No significant difference in weights was observed in other organs.

2.2.2.3 Effects of ODA on serum TC, TG and HDL-C

Serum TC in hamsters fed LA, CLA and LN were significantly lower than that of the control, but TC in CLN group was not significantly different from that in the control. To be specific, TC of LA, CLA and LN were 15.0%, 24.2% and 24.2% lower than the control. There was no significant difference in TC among LA, CLA and LN (Table 2.5). Serum HDL-C was not significantly different among the groups. Non-HDL-C/HDL-C ratio in CLA and LN groups, but not that in LA and CLN was significantly lower than the control. No significant difference in serum TG was observed among the hamsters fed the five experimental diets.

Table 2.3

Body weight gain and food intake of the hamsters

	Control	LA	CLA	LN	CLN
Initial body weight (g)	112.5 ± 5.9	111.0 ± 6.6	110.0 ± 7.1	119.5 ± 6.9	123.0 ± 9.8
Final body weight (g)	147.0 ± 8.2	142.8 ± 7.4	140.1 ± 10.8	145.9 ± 5.4	142.5 ± 7.1
Food intake (g/day)	10.8 ± 0.1	10.1 ± 0.2	10.1 ± 0.1	10.9 ± 0.1	10.5 ± 0.2

Values are expressed as mean ± S.D. (n=10).

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Table 2.4

Weights of liver, kidney and heart of hamsters fed the control, LA, CLA, LN and CLN diets

	Control	LA	CLA	LN	CLN
Liver (g)	6.39 ± 0.64^{c}	$6.83 \pm 0.56^{b, c}$	$7.71 \pm 0.98^{a, b}$	$7.35 \pm 1.68^{a, c}$	7.63 ± 0.81^{a}
Epididymal adipose tissue (g)	2.36 ± 0.38^{a}	2.27 ± 0.34^{a}	1.99 ± 0.30^{b}	2.77 ± 0.70^{a}	2.50 ± 0.74^{a}
Perirenal adipose tissue (g)	1.42 ± 0.20^{a}	1.45 ± 0.16^{a}	1.07 ± 0.22^{b}	1.31 ± 0.46^{a}	1.76 ± 0.69^{a}
Kidneys (g)	$1.11 \pm 0.08^{b, c}$	$1.12 \pm 0.11^{a, b}$	$1.19 \pm 0.11^{a, b}$	1.23 ± 0.14^{a}	$1.18 \pm 0.13^{a,b}$
Heart (g)	0.53 ± 0.08	0.53 ± 0.09	0.53 ± 0.05	0.54 ± 0.07	0.53 ± 0.07

Values are expressed as mean ± S.D. (n=10).

Means at the same row with different superscripts (a, b and c) differ significantly at $p<0.05$.

Table 2.5

Effect of octadecaenoic acid (ODA) feeding on plasma triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol (nHDL-C) and the ratio of nHDL-C to HDL-C of the hamsters at week 6.

	Control	LA	CLA	LN	CLN
TC (mg/ mL)	252.8 ± 34.6[a]	215.0 ± 36.0[b]	204.4 ± 35.8[b]	191.6 ± 37.5[b, c]	226.3 ± 31.3[a, c]
HDL-C (mg/ mL)	92.7 ± 15.6	84.9 ± 14.8	94.5 ± 17.1	81.9 ± 15.4	94.3 ± 14.2
nHDL-C (mg/ mL)	160.0 ± 28.9[a]	130.1 ± 27.3[b]	109.9 ± 30.0[b]	109.7 ± 32.3[b]	132.0 ± 26.1[a]
nHDL-C/ HDL-C	1.8 ± 0.5[a]	1.6 ± 0.4[a]	1.2 ± 0.4[b]	1.2 ± 0.2[b]	1.4 ± 0.3[a]
TG (mg/ mL)	247.8 ± 87.9	220.6 ± 92.6	215.8 ± 116.2	263.2 ± 135.0	243.4 ± 134.9

Values are expressed as mean ± S. D. (n=10).

nHDL-C were calculated by subtracting HDL-C from TC.

Means at the same row with different superscripts (a, b, c) differ significantly at $p<0.05$.

2.2.2.4 Effect of ODA on liver cholesterol

Liver cholesterol levels in hamsters fed LA, CLA, LN and CLN were all significantly lower than that in the control. Liver cholesterol was reduced the most in the CLA-fed group (65.8% reduction) , followed by that of LN (49.8%), CLN (36.6%) and LA (22.8%) (Figure 2.3).

When LA was compared with CLA, hamsters fed CLA had their liver cholesterol significantly lower than those fed LA. However, for LN and CLN, liver cholesterol of hamsters fed CLN was significantly higher than those fed LN.

2.2.2.5 Effect of ODA on fecal neutral sterol output

Concentration of neutral sterol in the feces of the hamsters at week 6 is shown in Figure 2. 4. Total neutral sterol content in the feces of hamsters fed CLA and LN but not LA and CLN was significantly higher than that in the control. Specifically, CLA increased fecal neutral sterol content by 53.7%. LN increased it by 21.7% while CLN had no significant effect on fecal neutral sterol content.

2.2.2.6 Effect of ODA on fecal acidic sterol output

Concentration of fecal acidic sterol output is shown in Figure 2.5. There was no significant difference in acidic sterol concentration in hamsters with or without ODA feeding.

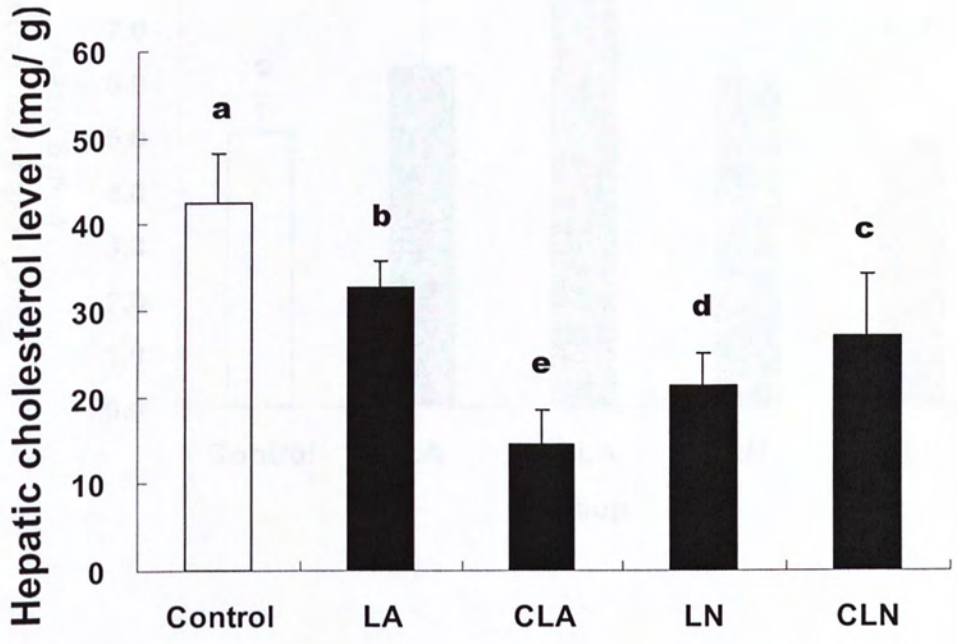

Figure 2.3

Effect of octadecaenoic acid (ODA) feeding on the liver cholesterol level in hamsters.

Values are expressed as means ± S.D. (n=10). Bars with different letters (a, b, c, d and e) are significantly different at $p<0.05$.

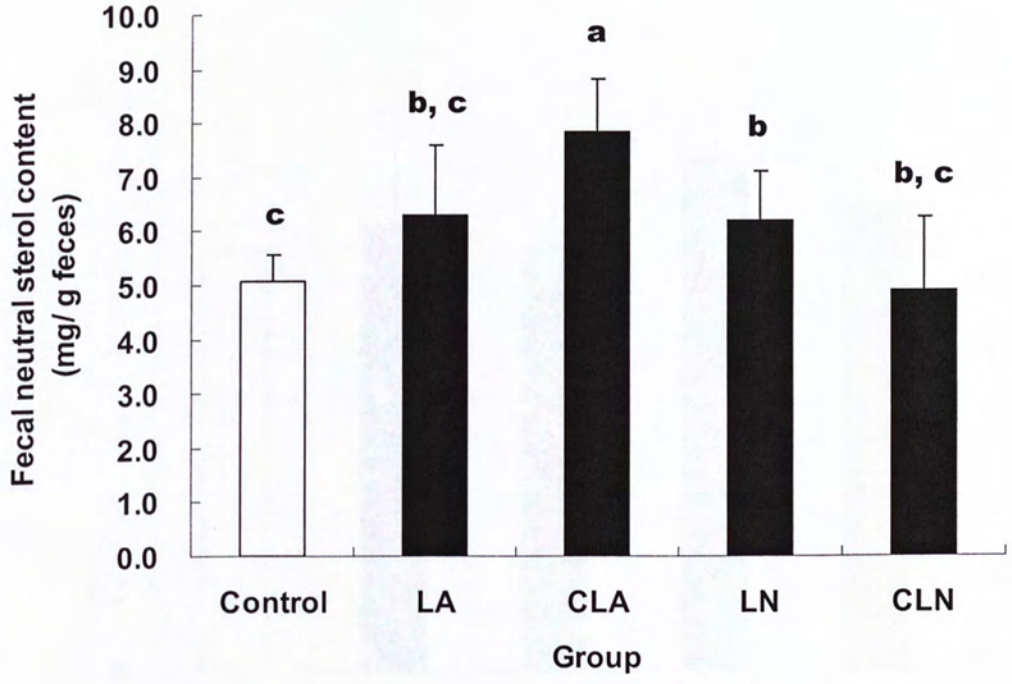

Figure 2.4

Effect of octadecaenoic acid (ODA) feeding on fecal neutral sterol output. Values are expressed as means ± S.D. (n=10). Bars with different letters (a, b and c) are significantly different at $p<0.05$.

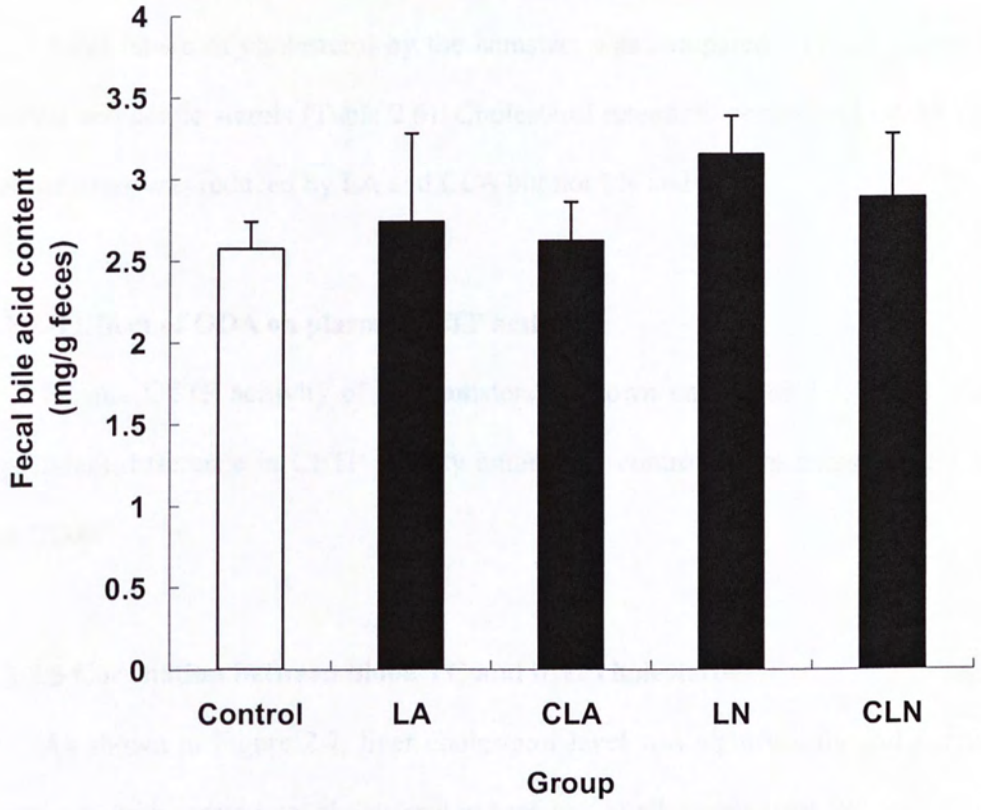

Figure 2.5

Effect of octadecaenoic acid (ODA) feeding on fecal acidic sterol output. Values are expressed as means ± S.D. (n=10).

2.2.2.7 Effect of ODA on cholesterol balance in hamsters

Total intake of cholesterol by the hamsters was compared with its excretion in neutral and acidic sterols (Table 2.6). Cholesterol retention/ cholesterol intake (%) in the hamsters was reduced by LA and CLA but not LN and CLN.

2.2.2.8 Effect of ODA on plasma CETP activity

Plasma CETP acitivity of the hamsters is shown on Figure 2.6. There was no significant difference in CETP activity among the control group hamsters and those fed ODA.

2.2.2.9 Correlation between blood TC and liver cholesterol

As shown in Figure 2.7, liver cholesterol level was significantly and positively correlated with serum total cholesterol in hamsters of all groups ($r=0.388$, $p<0.01$).

2.2.2.10 Correlation between blood HDL-C and liver cholesterol

As shown in Figure 2.8, liver cholesterol level had no significant association with serum HDL-cholesterol in hamsters of all groups ($r=0.031$, $p>0.05$).

2.2.2.11 Correlation between blood nHDL/HDL ratio and liver cholesterol

As shown in Figure 2.9, liver cholesterol level significantly and positively correlated with blood nHDL-C/ HDL-C ratio ($r=0.0455$, $p<0.05$) in hamsters of all groups .

Table 2.6

Dietary and fecal cholesterol balance in hamsters fed octadecaenoic acid (ODA) per day

	Control	LA	CLA	LN	CLN
Dietary cholesterol intake (mg)	9.50 ± 0.74	9.29 ± 0.51	9.21 ± 0.97	9.82 ± 0.36	10.07 ± 0.53
Fecal neutral sterol output (mg)	1.77 ± 0.27^{b}	2.14 ± 0.37^{b}	2.99 ± 0.60^{a}	2.15 ± 0.25^{a}	1.91 ± 0.70^{b}
Fecal acidic sterol output (mg)	0.95 ± 0.15	1.00 ± 0.10	0.95 ± 0.03	0.99 ± 0.15	1.02 ± 0.11
Cholesterol retained (mg)	6.97 ± 0.43	6.14 ± 0.66	5.46 ± 1.39	6.68 ± 0.30	7.14 ± 0.68
Cholesterol retained/ cholesterol intake (%)	73.6 ± 5.6^{a}	66.1 ± 4.5$^{b, c}$	59.0 ± 6.4^{c}	68.0 ± 2.8$^{a, b}$	71.0 ± 7.0$^{a, b}$

Values are expressed as mean ± S. D. (n=10). Means at the same row with different superscripts (a, b, c) differ significantly at $p<0.05$.

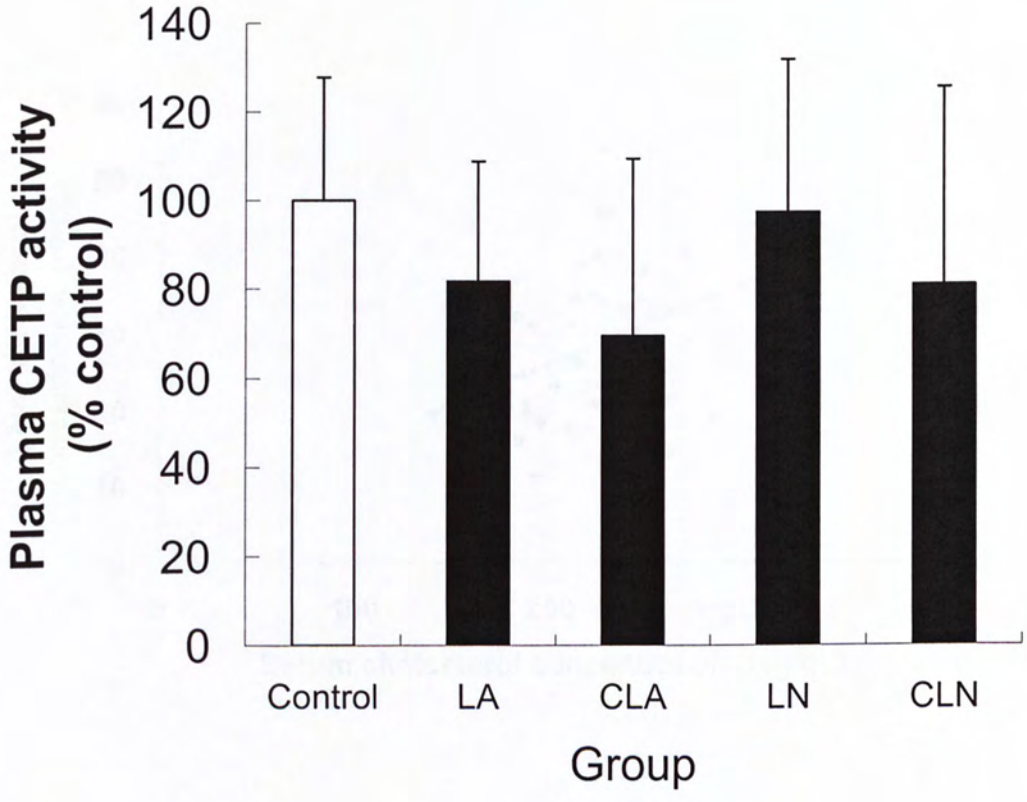

Figure 2.6

Plasma CETP activity in hamsters fed the control and octadecaenoic acid (ODA)-supplemented diets. Data are normalized so that CETP activity of the control group is regarded as 100%. Values are expressed as means ± S.D. (n=10).

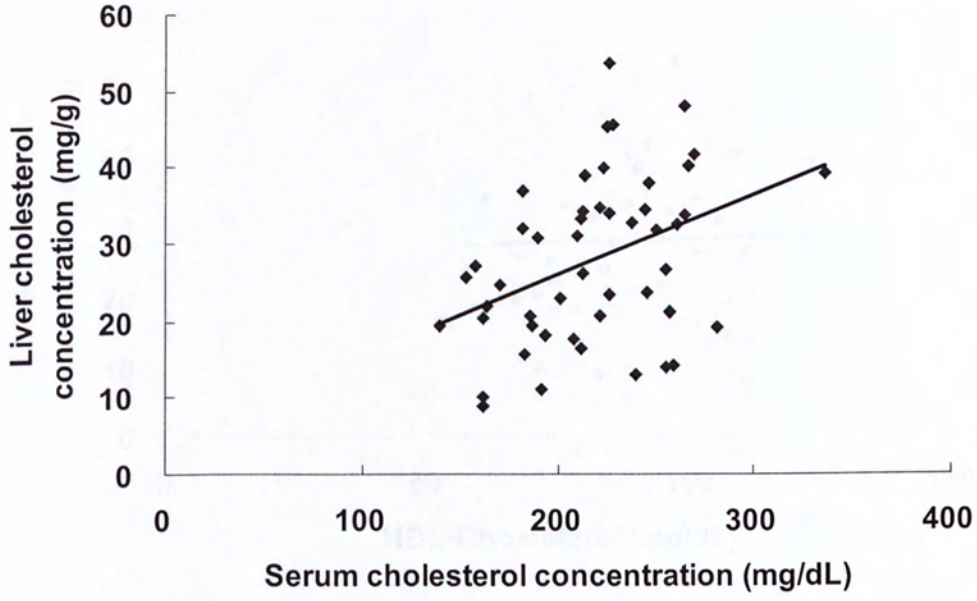

Figure 2. 7

Correlation between serum cholesterol and liver cholesterol levels, n=50, Pearson r=0.3883, $p<0.01$

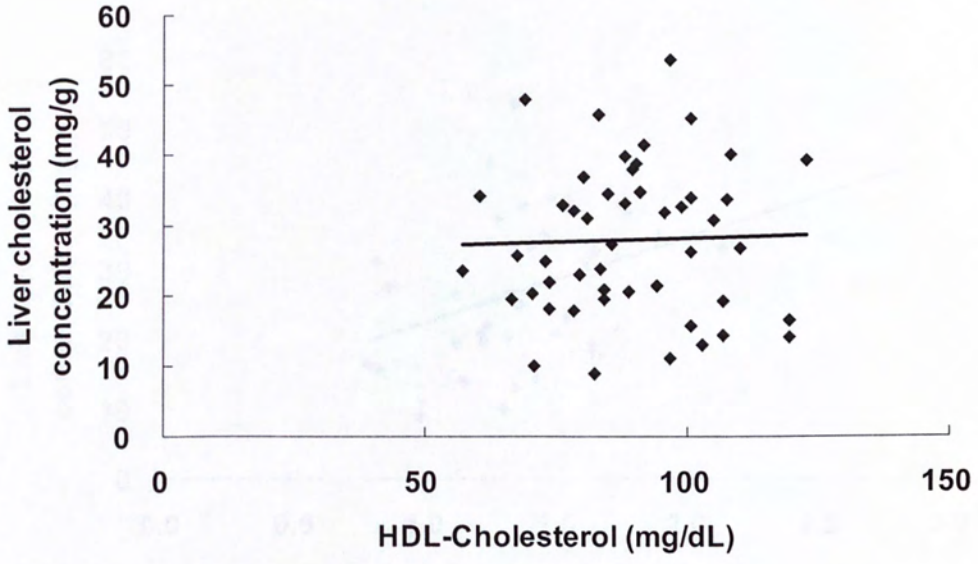

Figure 2.8

Correlation between serum HDL-cholesterol and liver cholesterol levels, n=50, Pearson r=0.031, p>0.05.

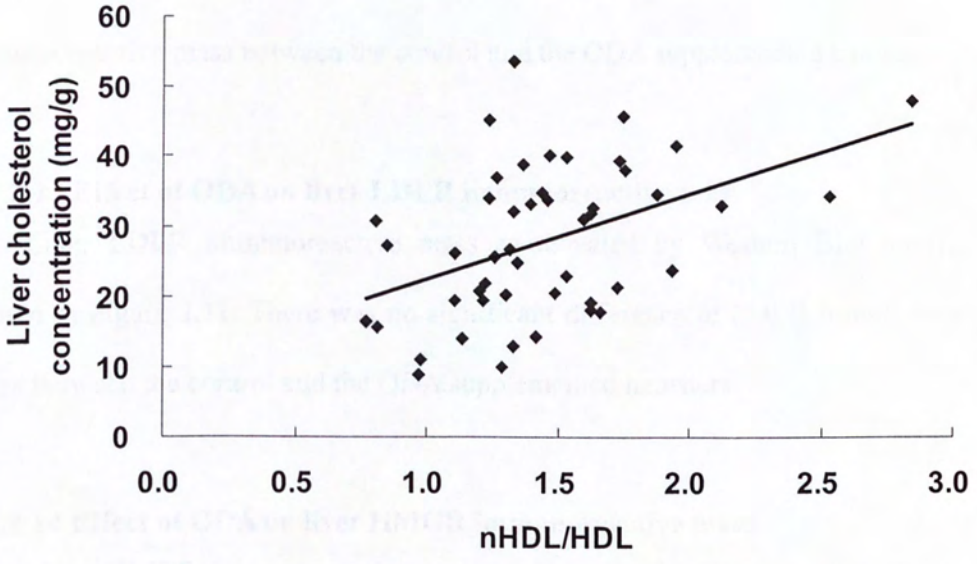

Figure 2.9

Correlation between serum non-HDL-cholesterol/ HDL-cholesterol ratio and liver cholesterol level, n=50, Pearson r=0.455, $p<0.01$.

2.2.2.12 Effect of ODA on liver SREBP-2 immunoreactive mass

Liver SREBP-2 immunoreactive mass as revealed by Western Blot analysis is shown in Figure 2.10. There was no significant difference in SREBP-2 immunoreactive mass between the control and the ODA supplemented hamsters.

2.2.2.13 Effect of ODA on liver LDLR immunoreactive mass

Liver LDLR immunoreactive mass as revealed by Western Blot analysis is shown in Figure 2.11. There was no significant difference in LDLR immunoreactive mass between the control and the ODA supplemented hamsters.

2.2.2.14 Effect of ODA on liver HMGR immunoreactive mass

Liver HMGR immunoreactive mass as revealed by Western Blot analysis is shown in Figure 2.12 There was no significant difference in HMGR immunoreactive mass between the control and the ODA supplemented hamsters.

2.2.2.15 Effect of ODA on liver LXR immunoreactive mass

Liver LXR immunoreactive mass as revealed by Western Blot analysis is shown in Figure 2.13. There was no significant difference in LXR immunoreactive mass between the control and the ODA supplemented hamsters.

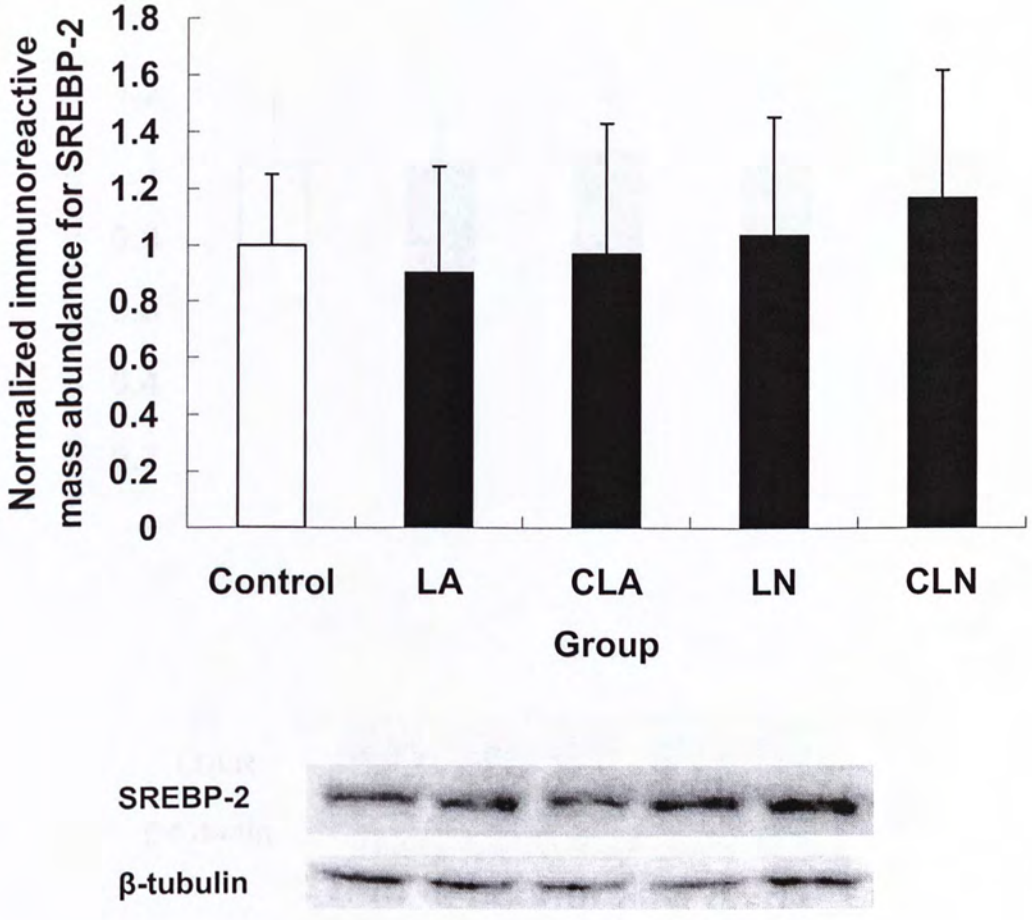

Figure 2.10

Relative abundance of hepatic sterol regulatory element-binding protein-2 (SREBP-2) immunoreactive mass as determined by Western Blot anaylsis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

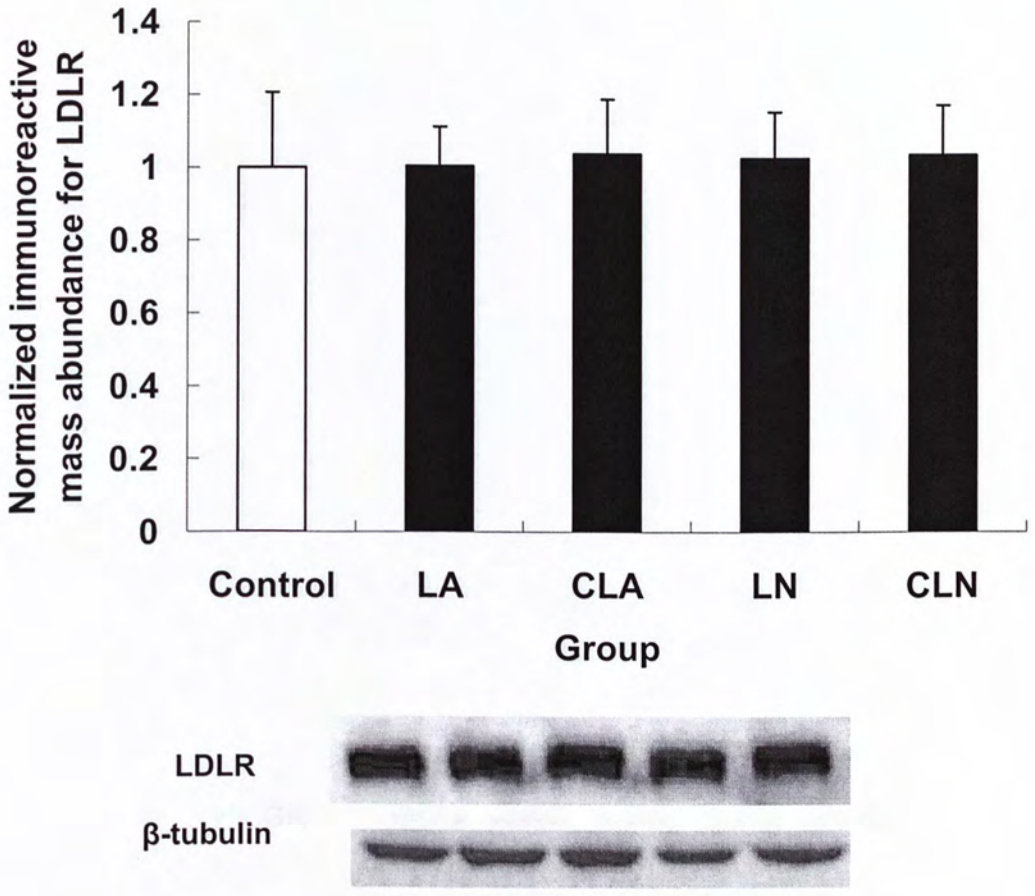

Figure 2.11

Relative abundance of hepatic low-density lipoprotein receptor (LDLR) immunoreactive mass as determined by Western Blot anaylsis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

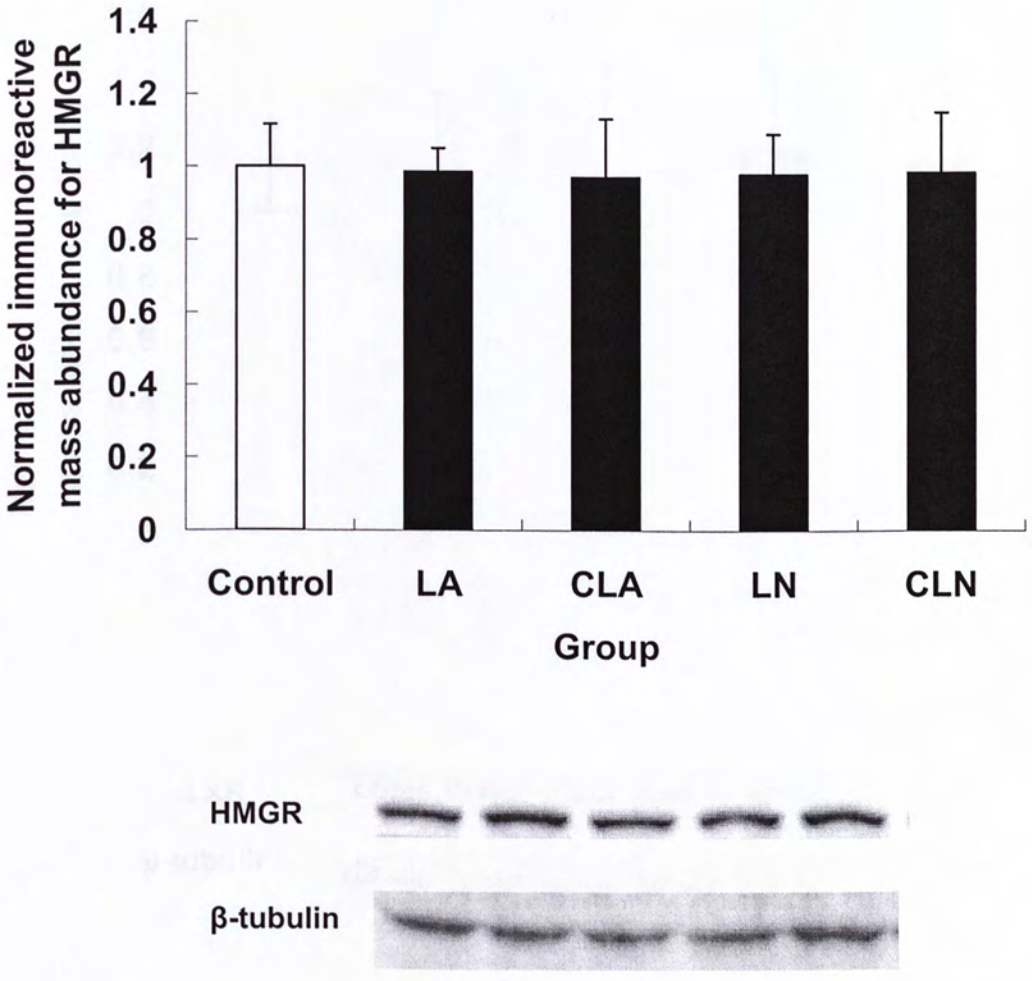

Figure 2.12

Relative abundance of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) immunoreactive mass as determined by Western Blot anaylsis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

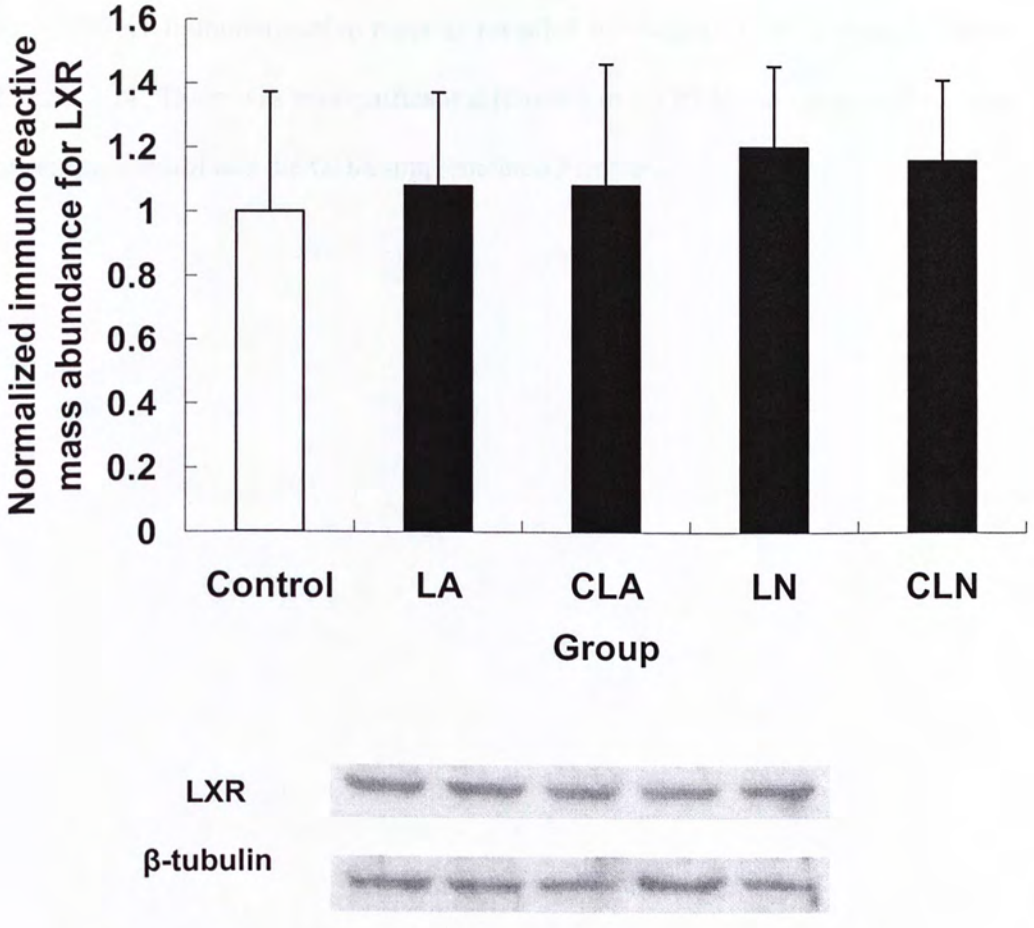

Figure 2.13

Relative abundance of hepatic liver-X-receptor (LXR) immunoreactive mass as determined by Western Blot anaylsis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

2.2.2.16 Effect of ODA on liver CYP7A1 immunoreactive mass

Liver CYP7A1 immunoreactive mass as revealed by Western Blot analysis is shown in Figure 2.14. There was no significant difference in CYP7A1 immunoreactive mass between the control and the ODA supplemented hamsters.

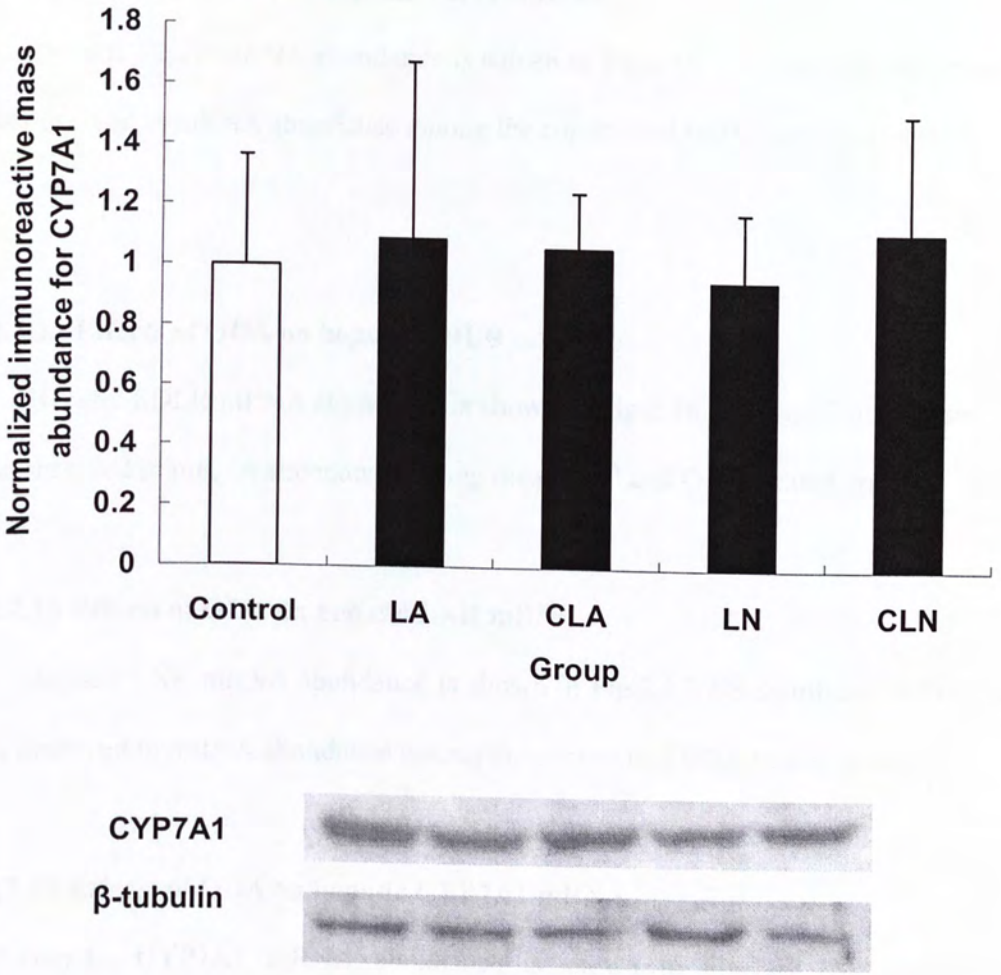

Figure 2.14

Relative abundance of hepatic cholesterol 7α-hydroxylase (CYP7A1) immunoreactive mass as determined by Western Blot anaylsis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

2.2.2.17 Effects of ODA on hepatic CETP mRNA

Hepatic CETP mRNA abundance is shown in Fig.2.15. No significant difference was observed in mRNA abundance among the control and ODA treated groups.

2.2.2.18 Effects of ODA on hepatic LDLR mRNA

Hepatic LDLR mRNA abundance is shown in Fig.2.16. No significant difference was observed in mRNA abundance among the control and ODA treated groups.

2.2.2.19 Effects of ODA on hepatic LXR mRNA

Hepatic LXR mRNA abundance is shown in Fig.2.17. No significant difference was observed in mRNA abundance among the control and ODA treated groups.

2.2.2.20 Effects of ODA on hepatic CYP7A1 mRNA

Hepatic CYP7A1 mRNA abundance is shown in Fig.2.18. No significant difference was observed in mRNA abundance among the control and ODA treated groups.

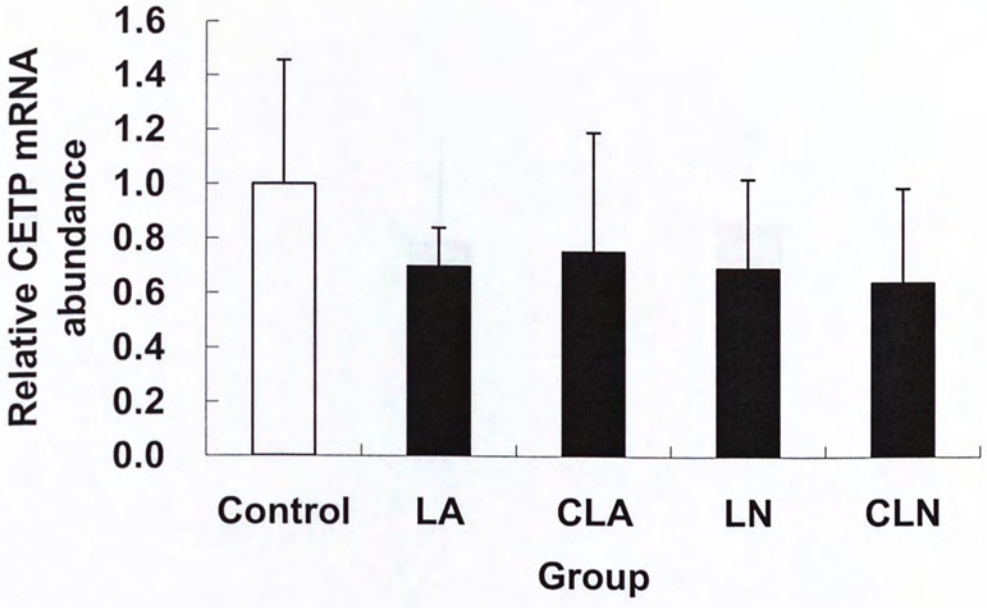

Fig 2.15

Hepatic cholesterol ester transfer protein (CETP) mRNA abundance in hamsters fed octadecaenoic acid (ODA). Data are normalized with GAPDH RNA so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

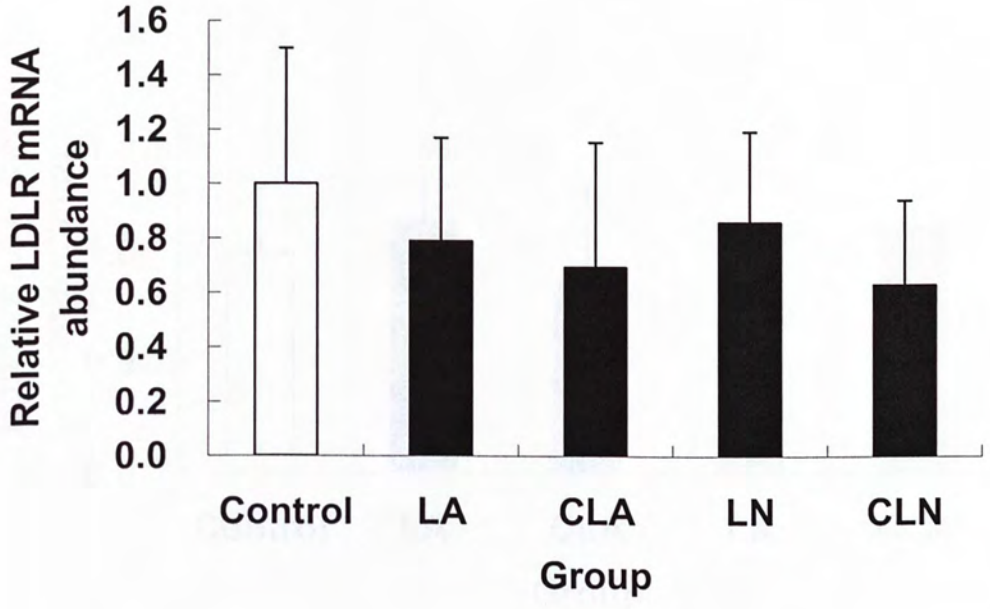

Fig 2.16

Hepatic low-density lipoprotein receptor (LDLR) mRNA abundance in hamsters fed octadecaenoic acid (ODA). Data are normalized with GAPDH RNA so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

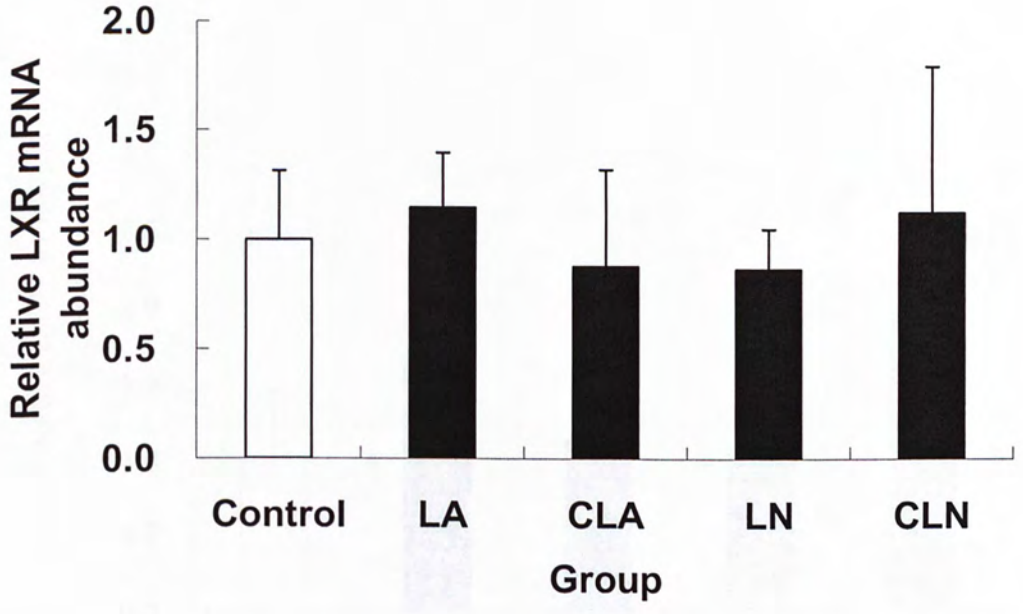

Fig 2.17

Hepatic liver-X-receptor (LXR) mRNA abundance in hamsters fed octadecaenoic acid (ODA). Data are normalized with GAPDH RNA so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

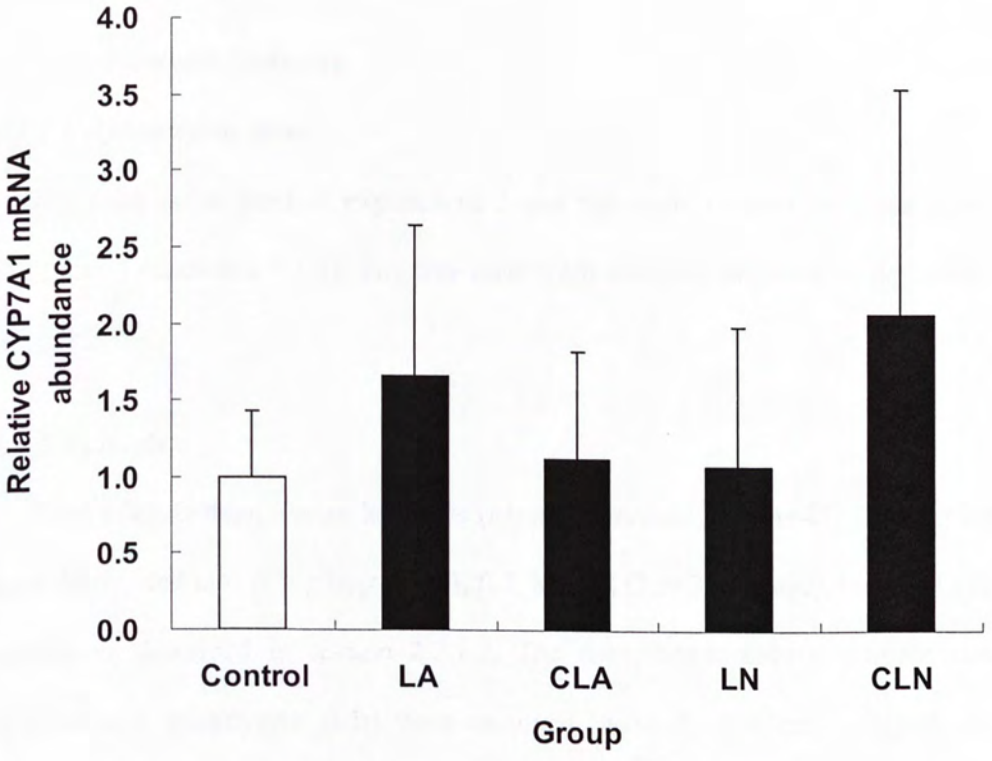

Fig 2.18

Hepatic cholesterol 7α-hydroxylase (CYP7A1) mRNA abundance in hamsters fed octadecaenoic acid (ODA). Data are normalized with GAPDH RNA so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=10).

2.3 Experiment 2

2.3.1 Materials and Methods

2.3.1.1 Experimental diets

The fatty acids used in experiment 2 had the same sources as those used in experiment 1 (section 2.2.1.1). The five diets were similarly prepared as described in section 2.2.1.3.

2.3.1.2 Animals

Male adult Golden Syrian hamsters (*Mesocricetus auratus,* n=45, 113±7g) were randomly divided into five groups (n=9), fed, housed (2 or 3 per cage), bled and killed similarly as described in section 2.2.1.2. The liver, heart, kidney, adipose tissue (perirenal and epididymal pads) were removed, washed in saline, weighed, flash frozen in liquid nitrogen and stored at -80°C until analysis. The small intestine was taken out, the first 5 cm (duodenum) discarded, and the lumen of the next 30 cm (jejunum) was flushed with ice cold PBS for 3 times, opened longitudinally and blotted dry with a blotting paper. The mucosal layer was scrapped off from the epithelial layer with a glass slide on ice and transferred to 10 mL of ice cold homogenizing buffer consisted of 0.05 M Tris at pH 7.8 with Complete ® protease inhibitor cocktail (Roche), and stored at -80°C until analysis.

2.3.1.3 Intestinal acyl coenzyme A: cholesterol acyltransferase (ACAT) activity measurement

Intestinal ACAT activity was measured as described previously by Cadigan & Chang (1988), Chang *et al* (1998), Diczfalusy *et al* (1996), Heider *et al* (1983), and Zhang *et al* (2003).

2.3.1.3.1 Preparation of intestinal microsome

The mucosa sample was thawed and then homogenized in a homogenizer (Wheaton, San Diego, CA, U. S. A.) for 10 strokes on ice. The mixture was then centrifuged at 800 × g for 15 minutes at 4°C. The supernatant containing the cytosol was transferred to a new vial and the pellet containing cell debris was discarded. The ssupernatant was then centrifuged again was repeated at 800 × g for 15 minutes at 4°C. The resultant supernatant was then transferred to a polycarbonate centrifuge tube (Beckman) and centrifuged at 100,000 × g for 60 minutes at 4 °C. The supernatant was then discarded and the microsome pellet was resuspended in 1 mL homogenizing buffer (0.05 M Tris, 1 M KCl, with Complete ® protease inhibitor cocktail (Roche)). CHAPS (Sigma) was then added to the resuspension with constant mixing to final concentration of 2% (w/v). The resuspension was then considered as the microsome and stored at -80 °C until analysis. Aliquot of the microsome was taken for protein concentration using a commercial protein assay kit (Bio-rad) according to the manufacturer's instructions.

2.3.1.3.2 ACAT activity assay

Microsome was adjusted with solubilization buffer to a protein concentration of 1 mg/ 150 μL. 400 nmol of cholesterol in 45% (w/v) 2-hydroxypropyl β-cyclodextrin aqueous solution was added to 1 mg of microsome and incubated on ice for 30 minutes and then in a 37 °C water bath for 5 minutes to equalize temperature. The reaction was initiated by adding an assay reagent of 0.517 nmol of [^{14}C] oleoyl-Coenzyme A (0.03μCi) (Perkin-Elmer, Waltham, MA, U. S. A.), 7.483 nmol of non-radioactive oleoyl-Coenzyme A (Sigma) and 10 nmol of fatty acid-free bovine serum albumin (Sigma) and the reaction mixture was incubated in a 37 °C water bath

for 20 minutes. The reaction was then stopped by adding 4.8 mL chloroform: methanol mixture (2:1, v/v) and 1mL saline and then chilling on ice. After the addition of 10 μg [^{3}H] cholesterol oleate (0.002 μCi) (as internal standard for loss corrrection), the mixture was centrifuged at 800 × g for 10 minutes at 4 °C and the lower organic layer was collected and transferred to a new tube and evaporated under a gentle nitrogen stream until dryness. 10 μg of cholesteryl oleate in 50 μL of chloroform was then added and the tube was vortexed thoroughly. The resuspension was then spotted on a thin-layer chromatography (TLC) plate (Merck, NJ, U. S. A.) and then developed in hexane: ethyl acetate: acetic acid (80:20: 1, v/v) for 45 minutes. After drying, the plate was stained in iodine vapor. The band corresponding to cholesterol oleate was cut off according to a cholesteryl oleate standard developed in parallel and transferred to a scintillation vial. 10 mL of OptiPhase HiSafe 2 scintillation fluid (Perkin-Elmer) was added to the vial which was incubated with agitation overnight. Radioactivity was then measured in an LS 6500 scintillation counter (Beckman) and the data was calculated based on [^{3}H] recovery.

2.3.2 Results

2.3.2.1 Growth and food intake

The body weight and food intake of the hamsters are shown in Table 2.7. No significant difference in amount of food intake or body weight was observed in the hamsters among the control, LA, CLA, LN or CLN groups.

2.3.2.2 Organ weights

Weights of liver, adipose tissues (epididymal and perirenal pads), kidneys and hearts of the hamsters are shown in Table 2.8.

Weights of liver of hamsters fed CLA were significantly higher than that of the hamsters fed the control diet. Weights of epididymal and perirenal adipose pads of hamsters fed CLA and perirenal adipose pad of LN were significantly lower than those fed the control diet. Kidneys of hamsters fed CLA weighed significantly higher than those fed the control diet. No significant difference in weights was observed in other organs.

2.3.2.3 Effect of ODA on serum TC, TG and HDL-C

Serum TC of hamsters fed LA, CLA and LN were significantly lower than that of the control, but TC of CLN group was not significantly different from that of the control. To be specific, TC of LA, CLA and LN were 15.0%, 24.2% and 24.2% lower than the control. There was no significant difference in TC among LA, CLA and LN. (Table 2.9) Serum HDL cholesterol were not significantly different among the groups. Non-HDL cholesterol /HDLcholesterol ratio of CLA and LN, but not LA and CLN were significantly lower than the control.

Table 2.7
Body weight gain and food intake of the hamsters

	Control	LA	CLA	LN	CLN
Initial body weight (g)	109.4±8.1	112.2±7.9	114.4±6.3	115.0±8.3	112.8±5.1
Final body weight (g)	136.7±9.7	136.7±6.1	125.0±7.5	127.8±9.7	133.9±8.6
Food intake (g/day)	9.67±0.21	9.60±0.27	9.84±0.53	9.58±0.26	9.39±0.35

Values are expressed as mean ± S.D. (n=9).

Table 2.8 Weights of liver, kidney and heart of hamsters fed the control, LA, CLA, LN and CLN diets.

	Control	LA	CLA	LN	CLN
Liver (g)	6.92±0.88 [b]	7.10±0.56 [b,]	7.86±0.36 [a]	6.76±1.08 [b]	7.03±0.68 [b]
Epididymal adipose tissue (g)	2.23±0.40 [a]	2.42±0.40 [a,]	1.89±0.19 [b, c]	2.21±0.49 [a, c]	2.02±0.53 [a, c]
Perirenal adipose tissue (g)	1.40±0.28 [a, b]	1.46±0.26 [a]	1.00±0.25 [c]	1.17±0.27 [b, c]	1.24±0.35 [a, b, c]
Kidneys (g)	1.17±0.06 [b]	1.22±0.04 [b]	1.31±0.06 [a]	1.22±0.10 [b]	1.21±0.04 [b]
Heart (g)	0.50±0.03	0.48±0.02	0.47±0.03	0.48±0.03	0.49±0.03

Values are expressed as mean ± S.D. (n=9).

Means at the same row with different superscripts (a, b, c) differ significantly at $p<0.05$.

Table 2.9

Effect of octadecaenoic acid (ODA) feeding on plasma triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol (nHDL-C) and the ratio of nHDL-C to HDL-C of the hamsters at week 6.

	Control	LA	CLA	LN	CLN
TC (mg/ mL)	227.2±16.8 [a]	200.8±31.5 [b]	183.4±36.5 [b]	172.5±25.0 [b]	222.5±27.9 [a]
HDL-C (mg/ mL)	109.1±7.5	101.5±4.8	101.4±14.2	94.8±11.4	102.3±11.8
nHDL-C (mg/ mL)	118.1±15.9 [a]	99.3±27.5 [b]	82.0±25.0 [b]	77.8±16.2 [b]	120.3±31.5 [a]
nHDL-C/ HDL-C	1.09±0.17 [a]	0.98±0.24 [a]	0.80±0.17 [b]	0.82±0.14 [b]	1.07±0.19 [a]
TG (mg/ mL)	195.7±49.5 [a]	141.5±24.4 [b,d]	91.7±19.2 [c]	114.0±38.1 [c,d]	174.1±57.5 [a,b]

Values are expressed as mean ± S. D. (n=9).

nHDL-C were calculated by subtracting HDL-C from TC.

Means at the same row with different superscripts (a, b, c) differ significantly at $p<0.05$.

No significant difference in serum TG was observed among the hamsters fed the five experimental diets.

2.3.2.4 Effect of ODA feeding on fecal neutral sterol content

Fecal neutral sterol content of the hamsters fed CLA and LN was significantly higher than the control, while that of hamsters fed LA and CLN was not significantly different (Figure 2.19).

2.3.2.5 Effect of ODA feeding on fecal acidic sterol content

Concentration of fecal acidic sterol output is shown in Figure 2.20. There was no significant difference in acidic sterol levels in hamsters with or without ODA feeding.

2.3.2.6 Effect of ODA feeding on intestinal acyl coenzyme A: acyl cholesterol transferase (ACAT) activity

The intestinal ACAT activity of hamsters fed CLA and LN, but not LA or CLN, was significantly lower than that of the control (Figure 2.21). Hamsters fed LA had their intestinal ACAT activity reduced by 63.6% compared with the control while those fed LN had a 41.1% reduction.

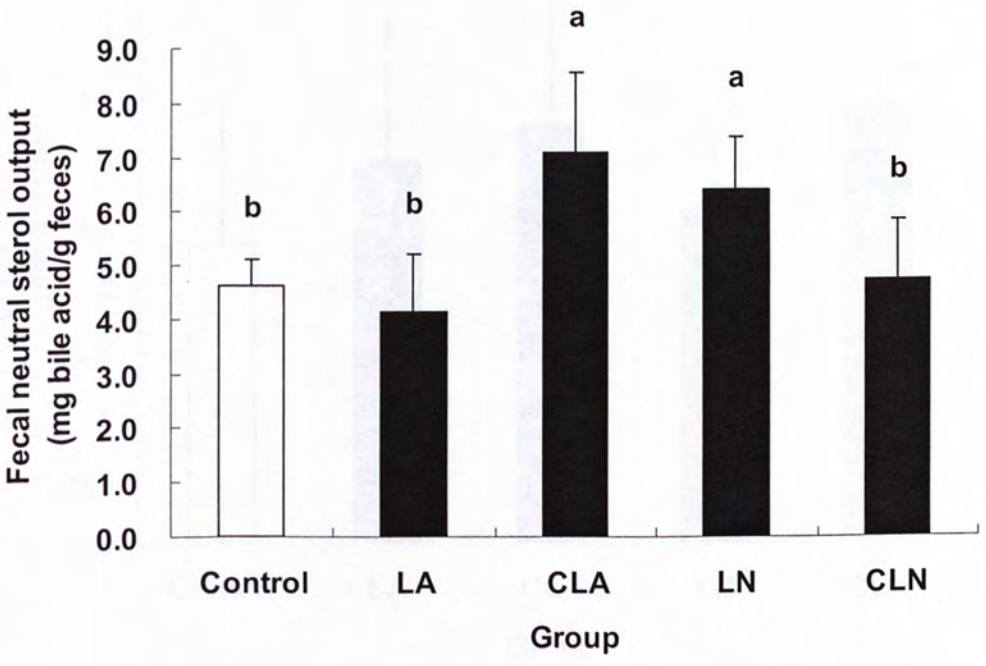

Figure 2.19 Effect of octadecaenoic acid (ODA) feeding on fecal neutral sterol content. Values are expressed as means ± S.D. (n=10). Bars with different letters (a, b and c) are significantly different at $p<0.05$.

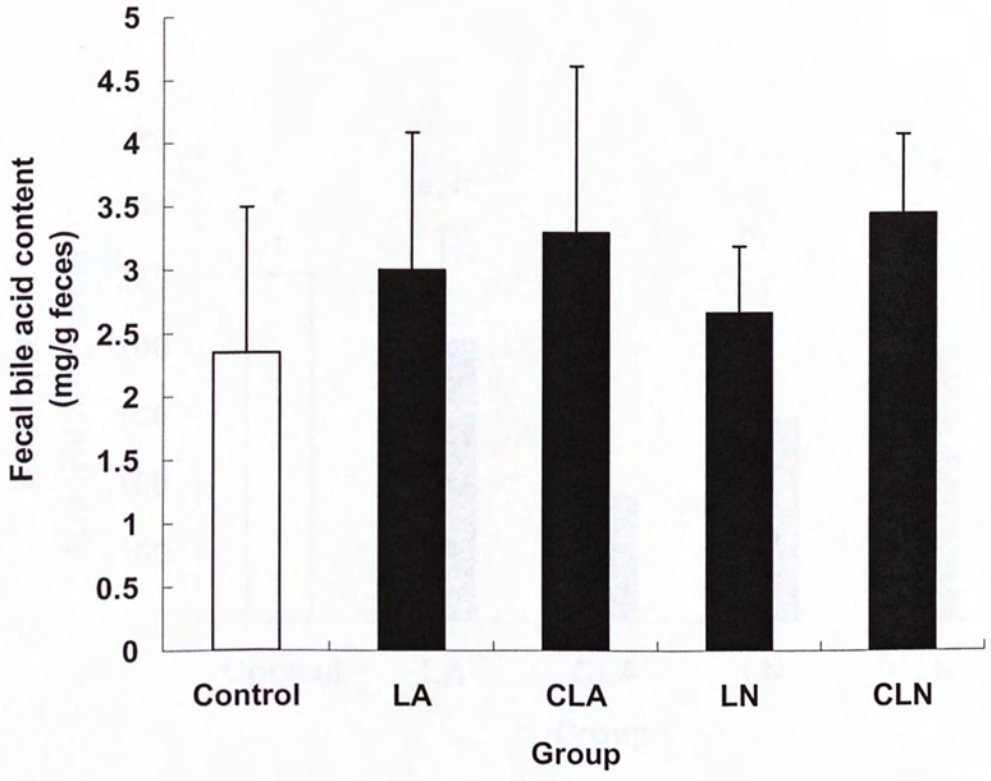

Figure 2.20 Effect of octadecaenoic acid (ODA) feeding on fecal acidic sterol content. Values are expressed as means ± S.D. (n=10).

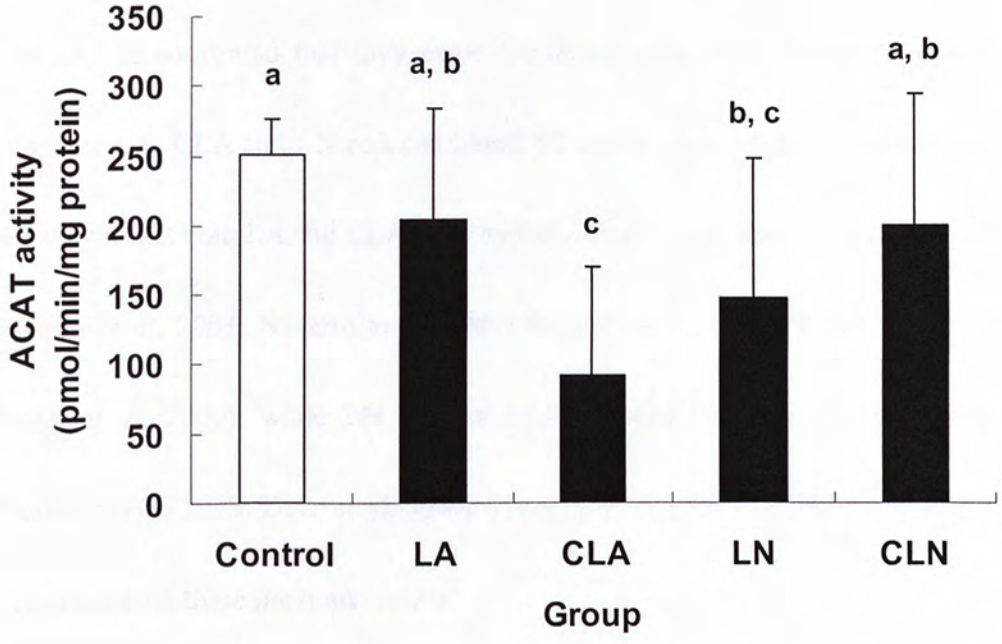

Figure 2.21 Intestinal acyl coenzyme A: acyl cholesterol transferase (ACAT) activity in hamsters fed LA, CLA, LN and CLN. Values are expressed as means ± S.D. (n=10). Bars with different letters (a, b and c) are significantly different at $p<0.05$.

2.4 Discussion

LA, CLA, LN and CLN are four structurally similar PUFAs. Experiments 1 and 2 clearly demonstrated that they exerted different effects on blood cholesterol in hamsters. LA, CLA and LN reduced blood TC while CLN did not. Previous studies had shown that both LA and CLA were hypocholesterolemic (de Deckere *et al*, 1999; Navarro *et al*, 2003; Navarro *et al*, 2005; Navarro *et al*, 2006; Wilson *et al*, 2006; Yeung *et al*, 2000), while LN but not CLN reduced blood cholesterol (Dhar & Bhattacharyya, 1998; Dhar *et al*, 1999; Yeung *et al*, 2000). The present results is in agreement with these previous reports.

In experiment 1, while reduction in TC was observed in the hamsters fed LA, CLA and LN, no change in HDL-C was observed among these groups. Therefore, reduction in cholesterol in serum occurred mainly in the non-HDL lipoprotein class. In fact, nHDL-C/HDL-C ratio was also significantly lower in CLA and LN groups than in the control group. It is known that LDL-C/HDL-C ratio is a strong predictor of CHD incidence. Therefore, dietary supplementation of LA, CLA and LN induces favorable change in the distribution of lipoproteins.

Blood TG responded to ODA supplementation in an inconsistent manner. In this study, no significant difference in TG was observed in the ODA-fed and the control hamsters. However, Dhar and Bhattacharyya (1999) reported an increase in TG in rats

fed CLN while Navarro (2006) observed an decrease in TG in hamsters fed CLN.

Yeung (2000) had shown that CLA but not LA increased fecal neutral sterol output with bile acid output being unaffected. In this study, we confirmed that CLA elevated fecal neutral sterol content and also showed that LN but not CLN increased fecal neutral sterol output. This suggests that LN and CLA share the property of increasing neutral sterol excretion in the feces. Increase in neutral sterol content suggests a lower efficiency of cholesterol absorption in the intestine.

Absorption of cholesterol was the lowest in the CLA-fed hamsters among the five groups (Table 2.5). Similarly, cholesterol absorption was significantly reduced in hamsters fed LA. In contrast, cholesterol absorption in the LN group seemed to be lower than the control, although the difference was not statistically significant. The present cholesterol balance study demonstrated clearly that the varying effects of ODA on blood cholesterol were associated with their effect on absorption of dietary cholesterol.

Hepatic cholesterol levels in all ODA-treated hamsters were lower than that in the control. This was in agreement with previous reports (Navarro *et al*, 2006; Wilson *et al*, 2006). Interestingly, liver cholesterol level was significantly and positively associated with blood TC and nHDL-C/HDL-C ratio (Figures 2.7 and 2.9). High liver cholesterol was associated with not only high blood TC, but also non HDL-C. This

suggests that the liver plays an important role in the regulation of blood cholesterol and also distribution of cholesterol in different lipoprotein classes.

However, no significant difference was observed in the hepatic protein of SREBP-2, HMGR, LDLR, LXR, and CYP7A1 among the five groups. No significant difference was observed in the mRNA level of LDLR, LXR and CYP7A1. Current knowledge on protein and mRNA abundances of these proteins affected by PUFAs *in vivo* is limited. A previous study reported that CLA did not affect mRNA levels of SREBP-1a and SREBP-1c in hamsters fed an atherogenic diet (Zabala *et al*, 2004). Since theses proteins function as the regulation points of input and output of cholesterol in the liver, it is suggested that homeostatic regulation of cholesterol induced by ODAs did not occur at transcriptional or translational levels *in vivo* under the current experimental conditions. No significant difference was observed in plasma CETP activity and hepatic CETP mRNA among all groups. CETP regulates the distribution of cholesterol between HDL and LDL. There was no evidence that the reduction in non HDL-C in LA, CLA and LN groups were brought about by alternation in CETP activity.

In view of association between increased fecal cholesterol output and lower blood cholesterol, experiment 2 was conducted to further investigate whether reduction in cholesterol absorption was due to intestinal ACAT inhibition. The blood

lipid profile of the hamsters in experiment 2 showed the same trend as that in experiment 1. The results are in agreement with previous reports (de Deckere *et al*, 1999; Dhar & Bhattacharyya, 1998; Dhar *et al*, 1999; Navarro *et al*, 2003; Navarro *et al*, 2005; Navarro *et al*, 2006; Wilson *et al*, 2006; Yeung *et al*, 2000). TC in hamsters fed LA, CLA and LN diets but not CLN diet were significantly lower than the control. HDL-cholesterol was not affected by ODA supplementation while non-HDL-cholesterol was significantly lowered in hamsters fed LA, CLA and LN diet. Interestingly, significant decreases in blood TG levels were also observed in LA, CLA and LN fed hamsters, while a similar trend but no significant difference were observed in the hamsters in experiment 1. As for fecal neutral sterol, significantly higher output was observed in CLA and LN fed hamsters but not in those fed LA and CLN. In general, the results in experiment 1 were reproduced in experiment 2.

It was clearly demonstrated in experiment 2 that decrease in blood cholesterol in CLA and LN fed hamsters was associated with an increase in fecal neutral sterol excretion, and also a decrease in intestinal ACAT activity. This was in agreement with the previous report of our laboratory that CLA but not LA reduced intestinal ACAT activity in hamsters (Yeung *et al*, 2000). Esterification of cholesterol by ACAT is an essential and rate-limiting step in intestinal cholesterol absorption, and intestinal ACAT activity is thus associated with intestinal cholesterol absorption efficiency

(Haugen & Norum, 1976; Parini *et al*, 2004; Repa *et al*, 2004; Willner *et al*, 2003). Reduced ACAT activity and increased fecal neutral sterol excretion suggest a reduced intestinal neutral sterol absorption in CLA and LN fed hamsters. Reduced cholesterol absorption essentially reduces incorporation and influx of cholesterol into the body, thus counteracting the effect of cholesterol load from the experimental diet on blood cholesterol. Previous reports had also shown that ACAT inhibition by pharmaceutic agents led to a 60% decrease in blood cholesterol in rats (Roth *et al*, 1992).

In summary, we found that LA, CLA, LN and CLN had no effect on SREBP-2, HMGR, LDLR, LXR and CYP7A1 at both transcriptional and translational levels in hamsters. Neither association was found between plasma CETP activity and blood cholesterol distribution neither. However, the present studies clearly demonstrated hamsters fed LA, CLA and LN but not CLN diet had lower intestinal ACAT activities, which led to lower intestinal cholesterol absorption efficiencies. This can explain, at least in part, the underlying mechanism of why LA, CLA, LN but not CLN lowered blood TC and non-HDL cholesterol levels.

Chapter 3

Effect of Octadecaenoic Acids on Cholesterol-regulating Genes in HepG2

3.1 Introduction

3.1.1 HepG2 as a model of cholesterol regulation

The liver plays an important role in cholesterol metabolism *in vivo*. As described in Chapter 1, over 50% of cholesterol synthesis occurs in the liver, which also regulates the excretion of cholesterol from the body. The liver is therefore considered as the single most important organ in cholesterol metabolism (Dietschy *et al*, 1993; Kruit *et al*, 2006; Repa & Mangelsdorf, 2000; Yao *et al*, 2007). Cell lines from hepatocyte origins are therefore ideal models for the study of cholesterol metabolism *in vitro*.

HepG2 is a cell line derived from the hepatic carcinoma of a 15-year-old male Caucasian. It is a perpetual adherent and morphologically epithelial cell line. Its nature as a cancer cell line allows rapid proliferation and it has been a popular cell line chosen for the study of cholesterol homeostasis (Bejta *et al*, 2007; Morikawa *et al*, 2007; Qian *et al*, 2007; Shin *et al*, 2007). HepG2 has been shown to possess many liver-specific functions (Havekes *et al*, 1987). It has also been shown that many genes

involved in cholesterol synthesis and degradation are retained in HepG2, including SREBP-2 (Mullen *et al*, 2004), HMGR (Busch *et al*, 1990), apoA (Cuthbert *et al*, 1997), LDLR (Lovati *et al*, 2000; Yu-Poth *et al*, 2004)and CYP7A1 (Morikawa *et al*, 2007; Pandak *et al*, 1996). We have therefore chosen HepG2 as the cell culture model for the study of the effect of ODA on cholesterol metabolism.

3.1.2 Effect of polyunsaturated fatty acids (PUFAs) on cholesterol regulating genes in cultured cells

It remains unclear how individual fatty acids affect the mRNA and protein levels of genes involved in cholesterol metabolism. Some animal studies found that saturated fatty acids reduced LDLR activity, protein and mRNA levels while PUFAs had an opposite effect (Kurushima *et al*, 1995; Mustad *et al*, 1996; Woollett *et al*, 1992). However, some other studies found no effects on these genes brought about by the fatty acids (Hennessy *et al*, 1992; Ringseis *et al*, 2006a; Sorci-Thomas *et al*, 1989). Inconsistent results are also found in *in vitro* studies. Some researchers found that PUFAs increased LDLR mRNA levels while others found PUFAs increased LDLR activity but not mRNA (Lindsey *et al*, 1992; Srivastava *et al*, 1995; Yu-Poth *et al*, 2005).

The enzyme activity of HMGR, the rate-limiting enzyme in cholesterol synthesis,

was found to decrease in cells treated with long chain PUFAs (Vignikin *et al*, 1989). In another study, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found to down-regulate HMGR activity but not mRNA level in breast cancer cells MCF-7 (Duncan *et al*, 2005).

As for CYP7A1, inconsistent results were also observed. CYP7A1 mRNA and bile acid excretion were found to increase simultaneously in mouse fed fish oil (Berard *et al*, 2004). In another study, mice fed a saturated fatty acid-rich diet had their CYP7A1 decreased, along with increased blood and liver cholesterol compared with the control (Li *et al*, 2005). Interestingly, in the same study, mice fed a long chain polyunsaturated fatty acid-rich diet still had their CYP7A1 mRNA decreased but the bile acid excretion increased while blood and liver cholesterol were found to decrease (Li *et al*, 2005). To the best of our knowledge, there is to date no report on the effect of fatty acids on CYP7A1 of a cultured cell model.

Summing up the above literature review, the previous effects of individual fatty acids on the expressions of genes involved in cholesterol metabolism in cultured cells are limited and some are even conflicting. There are especially few reports on the effect of individual 18-C PUFAs on these cholesterol regulators. It is therefore of importance to investigate the effects of some selected ODA, namely, LA, CLA and LN on the genes involved in cholesterol regulation in cell culture model.

3.1.3 Objectives

In chapter 2, the mechanism by which LA, CLA and LN reduced blood cholesterol was not explained by the hepatic cholesterol-regulating genes *in vivo*. The objectives of the present study were to investigate the effect of ODA on the mRNA and protein abundances of these genes *in vitro* on a HepG2 model.

3.2 Materials and Methods

3.2.1 Cell culture

The cell culture experiment was performed in a protocol modified from Yu-poth *et al* (2004) and Rumsey *et al* (1995). HepG2 cell line was purchased from American Type Culture Collection (Manassas, VA, U. S. A.). The cells were routinely maintained in 75 cm^2 culture flask with RPMI-1640 medium (Gibco™, Invitrogen, CA, U. S. A.) supplemented with 10% (v/v) fetal bovine serum (Gibco™, Invitrogen), 100 U/mL penicillin (Gibco™, Invitrogen) and 100 μg/mL streptomycin (Gibco™, Invitrogen) in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. The medium was replenished every three days and the cells were passed to a new flask when the cells reached 70-80% confluent. During cell passage, the flask was washed with 5 mL of phosphate-buffered saline (PBS) and then incubated with 2 mL of EDTA-trypsin (Gibco™, Invitrogen) for 5 minutes in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. The detached cells were washed by 10 mL of PBS and centrifuged at 1500 rpm for 10 minutes. The pelleted cells were then resuspended in 2 mL of prewarmed fresh medium described above. 500 μL of the cell resuspension was transferred into a new 75 cm^3 culture flask containing 11.5 mL prewarmed medium.

For cell stock storage, the cell pellet obtained after trypsinzation and

centrifugation was resuspended with 3 mL of freezing medium containing 90% chilled fresh medium described above and 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma). 1 mL of the aliquot was transferred to a cryogenic vial (Corning, NY, Canada), which was then put in a cryogenic freezer (Corning) at -80°C overnight and then stored under liquid nitrogen.

To maintain a cell line from the stock, the cryogenic vial was taken from liquid nitrogen and allowed to thaw in a 37°C water bath until it just melted. The mixture was diluted with 10 mL of fresh medium and centrifuged at 1800 rpm for 5 minutes and the process is repeated once. The cells were finally resuspended in 2 mL of fresh medium and transferred into a 75 cm^2 culture flask containing 10 mL prewarmed fresh medium and incubated in a humidified atmosphere composed of 5 % CO_2 and 95% air at 37°C.

To study the effect of ODA-supplementation on HepG2, 1×10^5 of cells were seeded on a 100 mm^2 culture dish on day 0. On day 3, the medium was removed and the dish was rinsed with 5 mL PBS for three times, followed by the addition of a defined serum free medium consisting of RPMI-1640 (Gibco™, Invitrogen,), 100 U/mL penicillin (Gibco™, Invitrogen) and 100 μg/mL streptomycin (Gibco™, Invitrogen) and 1.5% (w/v) fatty acid–free BSA (Sigma) for 24 hours. On day 4, the medium was removed and replaced with the control medium or the

ODA-supplemented medium for 24 hours. After the incubation, the medium was removed and the dish was rinsed with 5 mL PBS for 3 three times and the cells were harvested for analysis.

To prepare the ODA-supplemented media, LA, CLA and LN were purchased from Sigma and were dissolved in 100% ethanol into LA, CLA and LN-stock solutions. The stock solutions were then slowly added below the surface-air interface to prevent dispersion of fatty acid over the surface, and were thoroughly dissolved in the defined serum free medium prewarmed to 37°C. The concentrations of BSA-bound LA, CLA and LN were adjusted to 0.4 mmol/L and that of ethanol was adjusted to 0.05% (v/v) in the experimental media. A concentration of 0.4 mmol/L of the BSA-bound fatty acid was determined by Yu-poth *et al* (2004) as a physiological concentration. The control medium was prepared by the addition of fatty acid-free BSA to a concentration of 1.5% (w/v) and ethanol to 0.05% (v/v) without the addition of ODA.

3.2.2 Measurement of SREBP-2, LDLR, HMGR and CYP7A1 protein abundance by Western blotting

Cellular protein was extracted in a method modified from Vaziri *et al* (1996). In brief, the cells were scrapped on ice with a cell scrapper in 2 mL of PBS (Corning),

followed by centrifugation at 1800 rpm for 5 minutes, and the supernatant above the cell pellet was aspirated and 200 μL of ice-cold homogenizing buffer (20mM Tris-HCl (pH 7.5), 2mM $MgCl_2$, 0.2 M sucrose and Complete ® protease inhibitor cocktail (Roche)) was added. After brief sonication, the samples were centrifuged at 12, 000 *g* for 15 minutes at 4 °C to remove cell debris. The supernatant was then saved, considered as the protein extract and stored at -20°C until analysis.

The remaining procedure for Western blotting was the same as described in Section 2.2.1.6, except that 25 μg of total protein was used in the detection of HMGR and LXR while 40μg was used for SREBP-2 and CYP7A1.

3.2.3 Measurement of cellular SREBP-2, LDLR, HMGR, LXR, CYP7A1 and CETP mRNA by real time PCR

RNA in the cells was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. In brief, 1 mL of Trizol was added into the culture dish and the cells were lyzed. The remaining procedures of RNA extraction, cDNA synthesis and real time PCR analysis were the same as described in chapter 2.

3.2.4 Statistics

Results were presented as means ± standard deviation (S.D.). Where applicable,

statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test, or Pearson's correlation coefficient, using Prism® (Graphpad software, Inc., CA, U. S. A.). Differences between groups were considered significant when $P<0.05$.

3.3 Results

3.3.1 Effect of ODA on HepG2 SREBP-2 immunoreactive mass

HepG2 SREBP-2 immunoreactive mass as revealed by Western blot analysis is shown in Figure 3.1. There was no significant difference in SREBP-2 immunoreactive mass between cells supplied with control and ODA supplemented media.

3.3.2 Effect of ODA on HepG2 HMGR immunoreactive mass

HepG2 HMGR immunoreactive mass as revealed by Western blot analysis is shown in Figure 3.2. There was no significant difference in HMGR immunoreactive mass between cells supplied with control and ODA supplemented media.

3.3.3 Effect of ODA on HepG2 LDLR immunoreactive mass

HepG2 LDLR immunoreactive mass as revealed by Western blot analysis is shown in Figure 3.3. There was no significant difference in LDLR immunoreactive mass between cells supplied with control and ODA supplemented media.

3.3.4 Effect of ODA on HepG2 LXR immunoreactive mass

HepG2 LXR immunoreactive mass as revealed by Western blot analysis is shown in Figure 3.4. CLA and LN supplementation significantly increased LXR

immunoreactive mass while there was no significant difference in LXR immunoreactive mass between control and LA-supplemented cells. More specifically, CLA and LN increased LXR immunoreactive mass by 12 and 9 % respectively.

3.3.5 Effect of ODA on HepG2 CYP7A1 immunoreactive mass

HepG2 CYP7A1 immunoreactive mass as revealed by Western blot analysis is shown in Figure 3.5. There was no significant difference in CYP7A1 immunoreactive mass between cells supplied with control and ODA supplemented media.

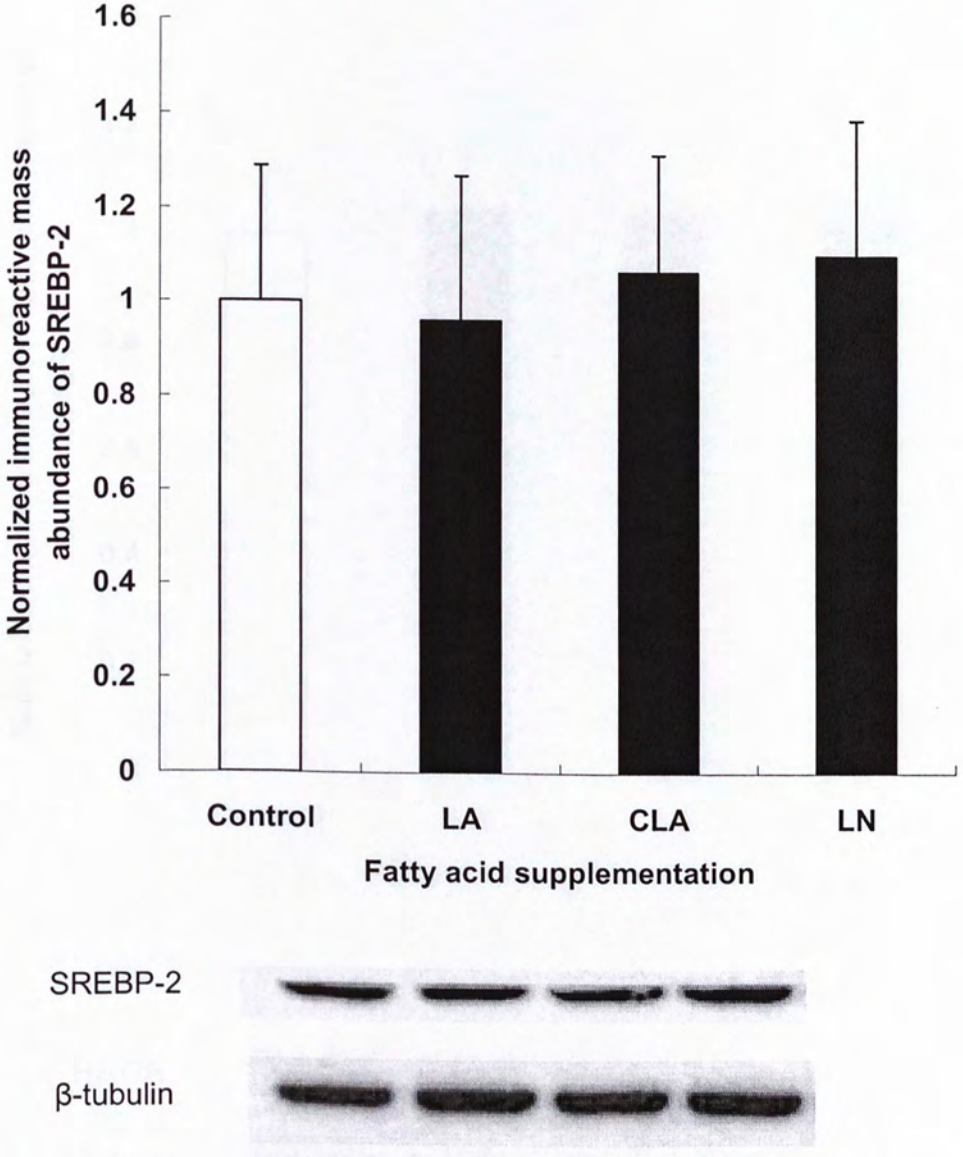

Figure 3.1 Relative abundance of HepG2 SREBP-2 immunoreactive mass as determined by Western blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

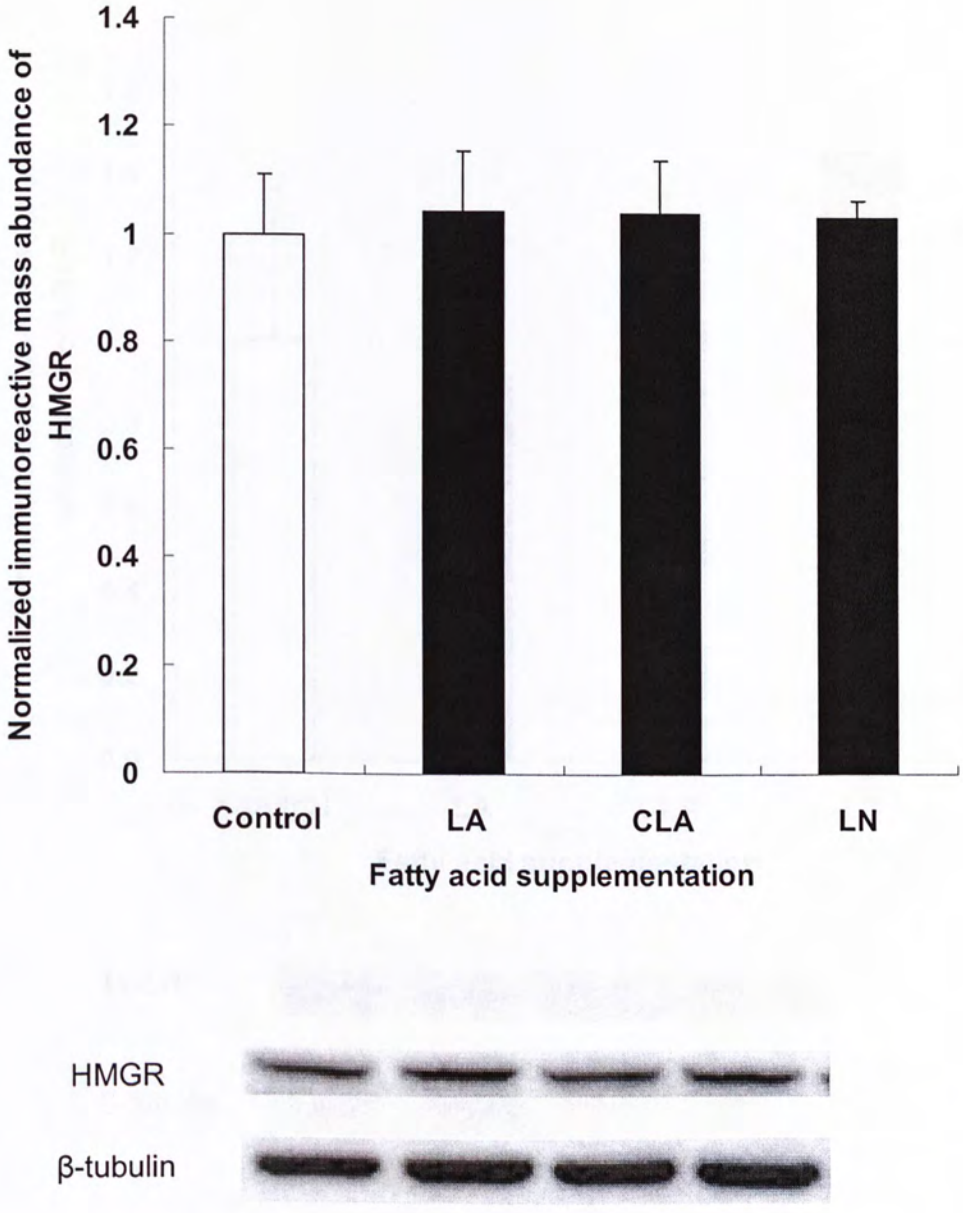

Figure 3.2 Relative abundance of HepG2 HMGR immunoreactive mass as determined by Western blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

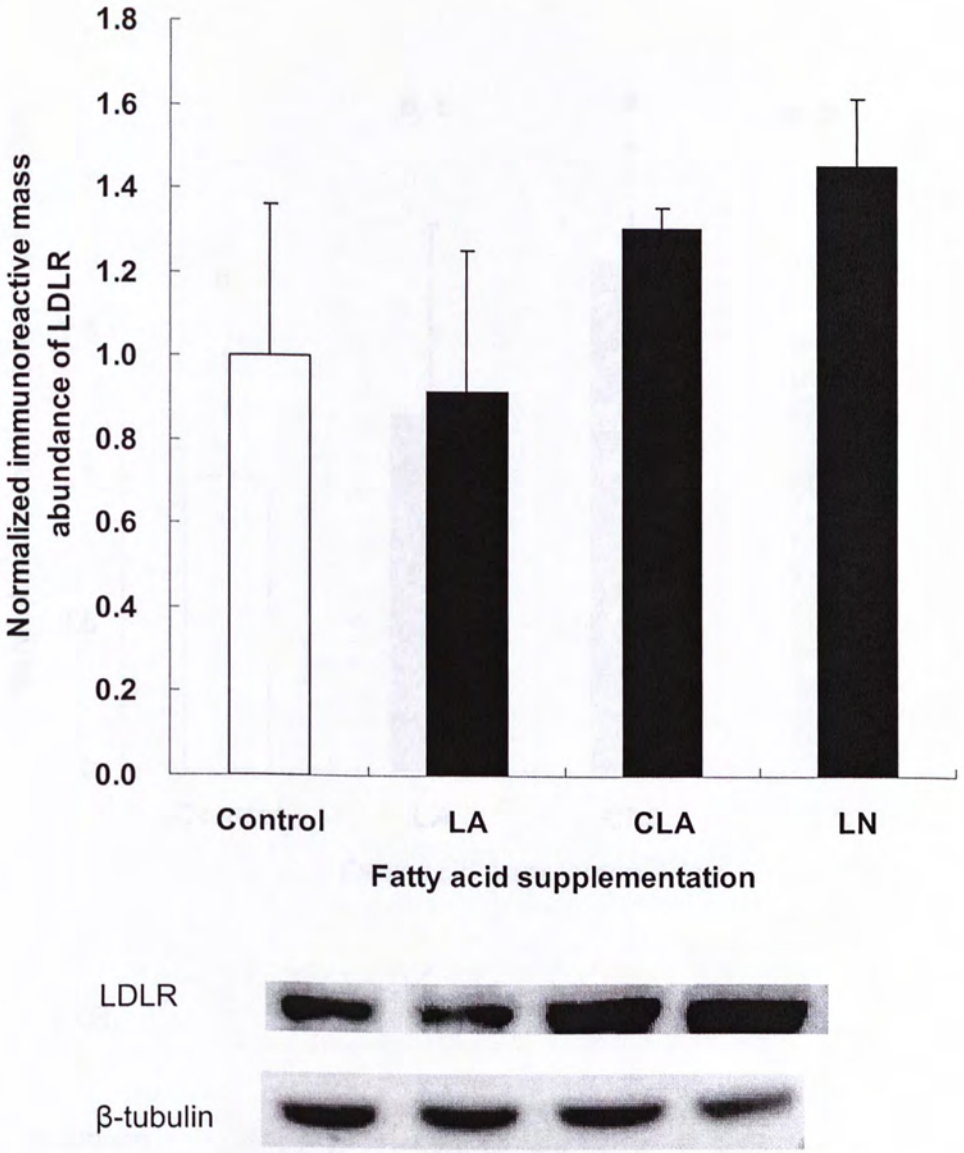

Figure 3.3 Relative abundance of HepG2 LDLR immunoreactive mass as determined by Western blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=3).

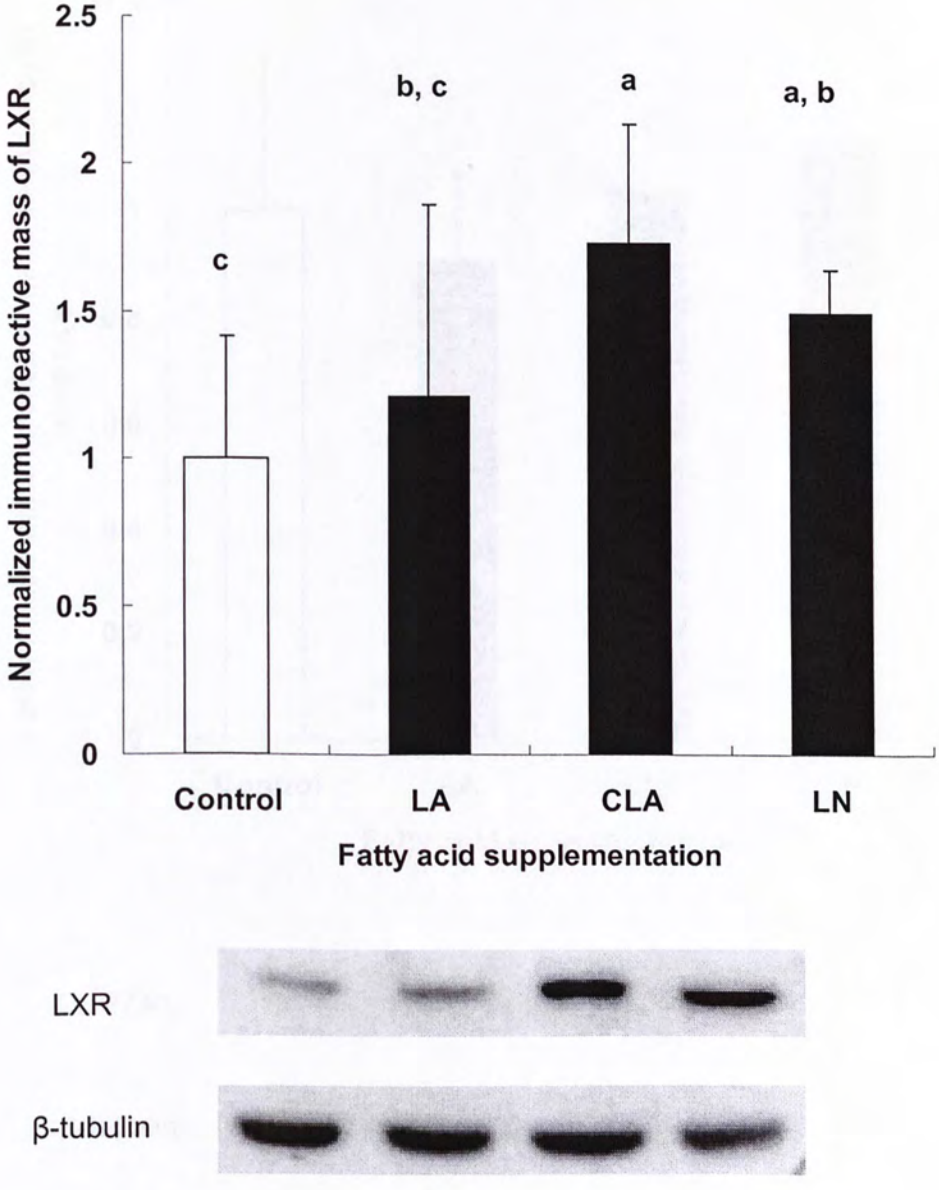

Figure 3.4 Relative abundance of HepG2 LXR immunoreactive mass as determined by Western blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

Means with different letters (a, b and c) differ significantly at $p<0.05$.

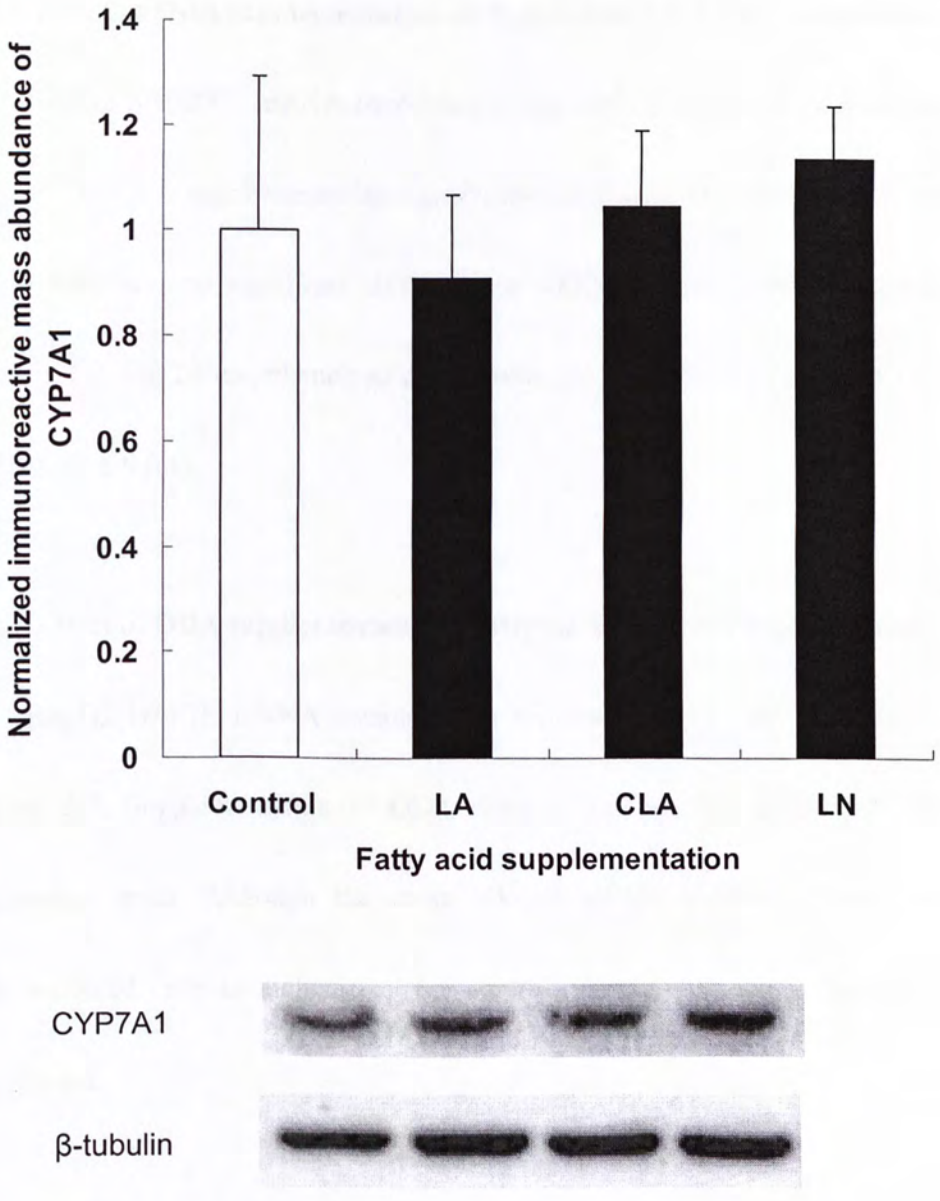

Figure 3.5 Relative abundance of HepG2 CYP7A1 immunoreactive mass as determined by Western blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

3.3.6 Effect of ODA supplementation on HepG2 SREBP-2 mRNA expression

HepG2 SREBP-2 mRNA abundance as revealed by real time PCR is shown in Figure 3.6. CLA supplementation significantly increased SREBP-2 gene expression while there was no significant difference in SREBP-2 gene expression among the control, LA and LN-supplemented cells. More specifically, CLA increased SREBP-2 mRNA by 5.6 fold.

3.3.7 Effect of ODA supplementation on HepG2 HMGR mRNA expression

HepG2 HMGR mRNA abundance as revealed by real time PCR is shown in Figure 3.7. Supplementation of ODA did not significantly change HMGR gene expression level. Although the mean HMGR mRNA expression level of CLA supplemented cells is higher than the control, the difference was not statistically significant.

3.3.8 Effect of ODA supplementation on HepG2 LDLR mRNA expression

HepG2 LDLR mRNA abundance as revealed by real time PCR is shown in Figure 3.8. CLA supplementation significantly increased LDLR gene expression while there was no significant difference in LDLR gene expression among the control, LA and LN-supplemented cells. More specifically, CLA increased LDLR mRNA by 7.8 fold.

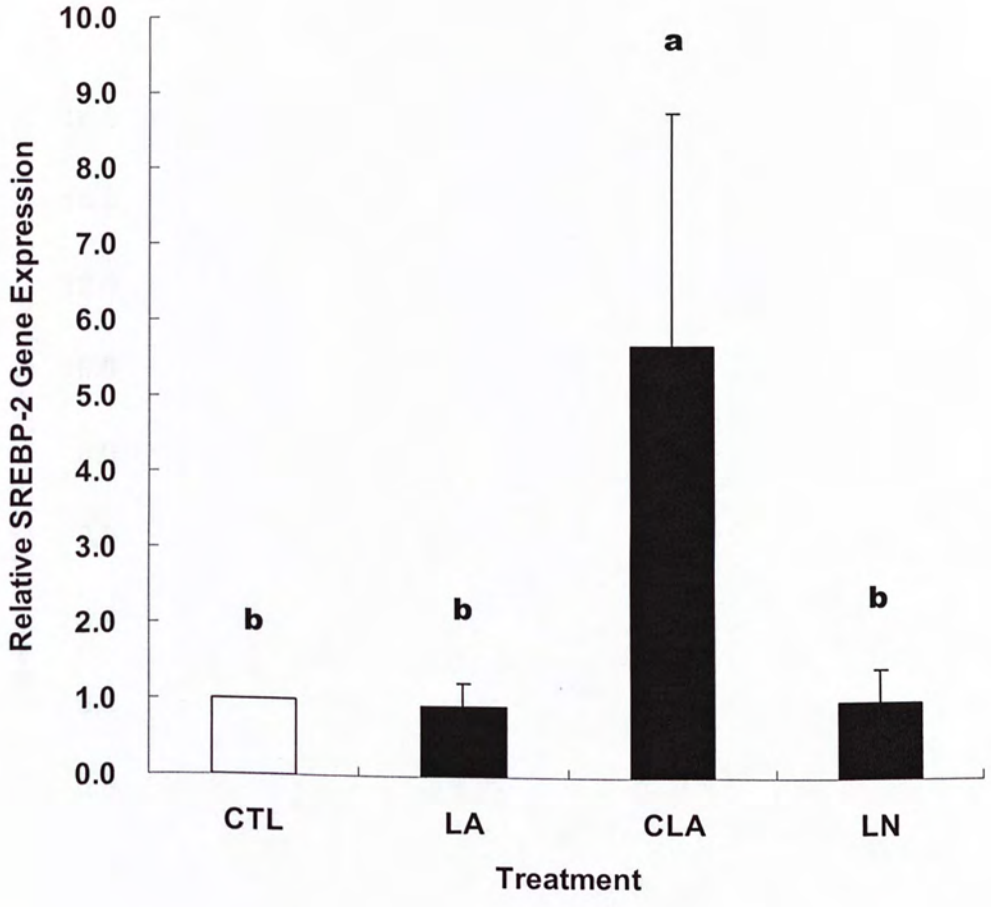

Figure 3.6 Relative SREBP-2 mRNA abundance in HepG2 as determined by real time PCR. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5). Means with different superscripts (a, b) differ significantly at $p<0.05$.

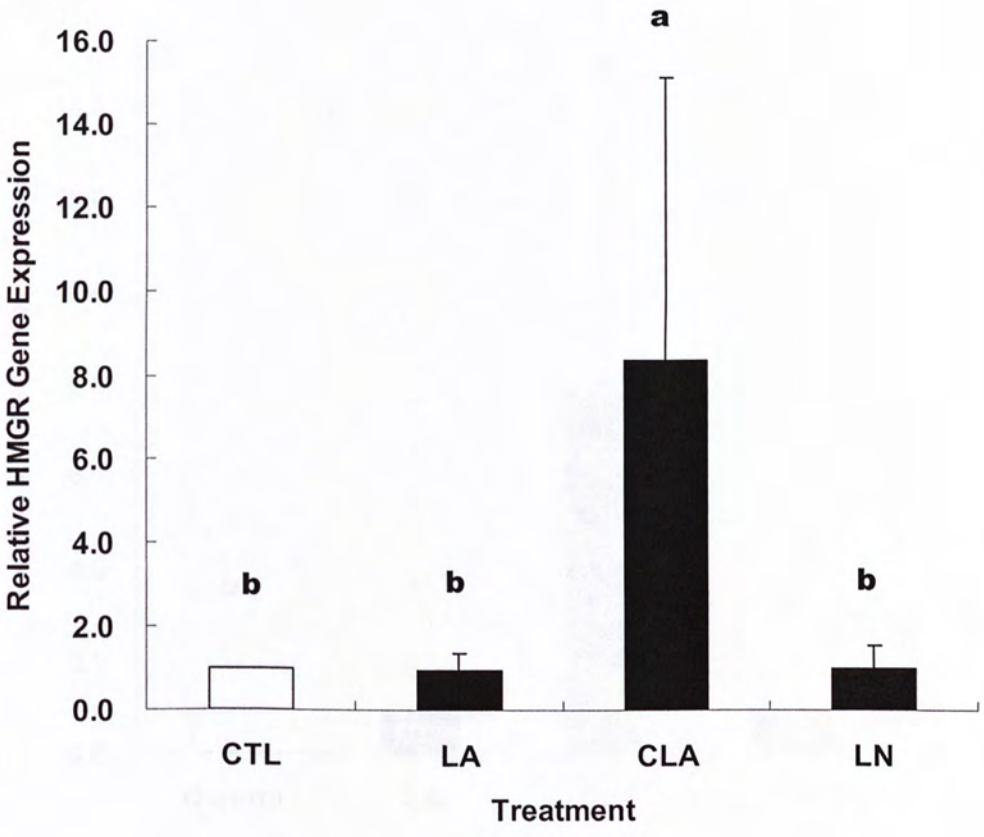

Figure 3.7 Relative HMGR mRNA abundance in HepG2 as determined by real time PCR. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

Means with different superscripts (a, b) differ significantly at $p<0.05$.

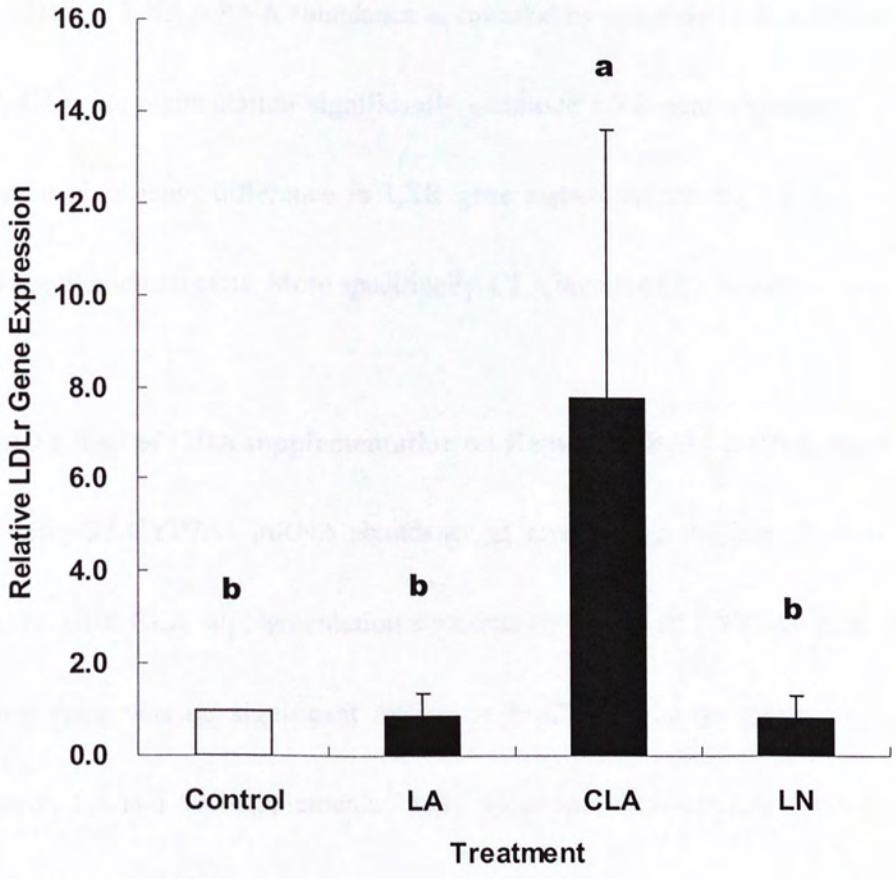

Figure 3.8 Relative LDLR mRNA abundance in HepG2 as determined by real time PCR. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

Means with different superscripts (a, b) differ significantly at $p<0.05$.

3.3.9 Effect of ODA supplementation on HepG2 LXR mRNA expression

HepG2 LXR mRNA abundance as revealed by real time PCR is shown in Figure 3.9. CLA supplementation significantly increased LXR gene expression while there was no significant difference in LXR gene expression among the control, LA and LN-supplemented cells. More specifically, CLA increased LXR mRNA by 5.5 fold.

3.3.10 Effect of ODA supplementation on HepG2 CYP7A1 mRNA expression

HepG2 CYP7A1 mRNA abundance as revealed by real time PCR is shown in Figure 3.10. CLA supplementation significantly increased CYP7A1 gene expression while there was no significant difference in CYP7A1 gene expression among the control, LA and LN-supplemented cells. More specifically, CLA increased CYP7A1 mRNA by 3.8 fold.

3.3.11 Effect of ODA supplementation on HepG2 CETP mRNA expression

HepG2 CETP mRNA abundance as revealed by real time PCR is shown in Figure 3.11. Supplementation of ODA did not significantly change CETP gene expression level. Although the mean CETP mRNA expression level of CLA supplemented cells is higher than the control, the difference was not statistically significant.

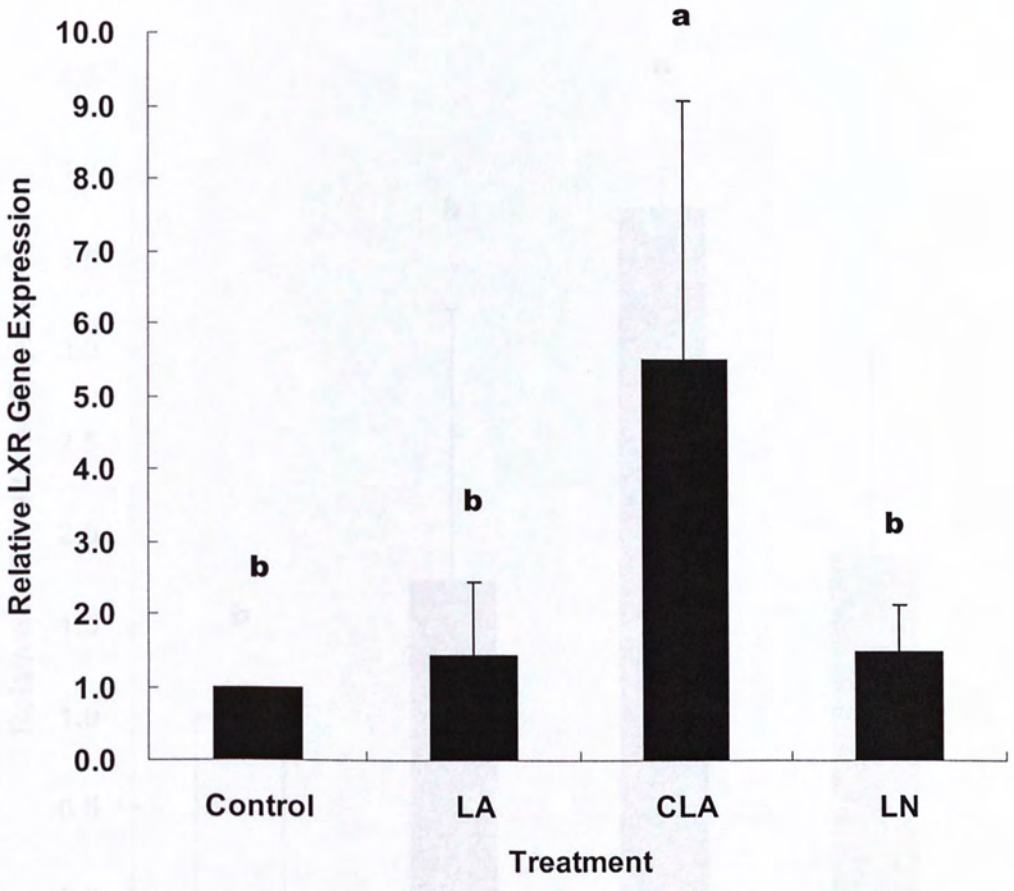

Figure 3.9

Relative LXR mRNA abundance in HepG2 as determined by real time PCR. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

Means with different superscripts (a, b) differ significantly at $p<0.05$.

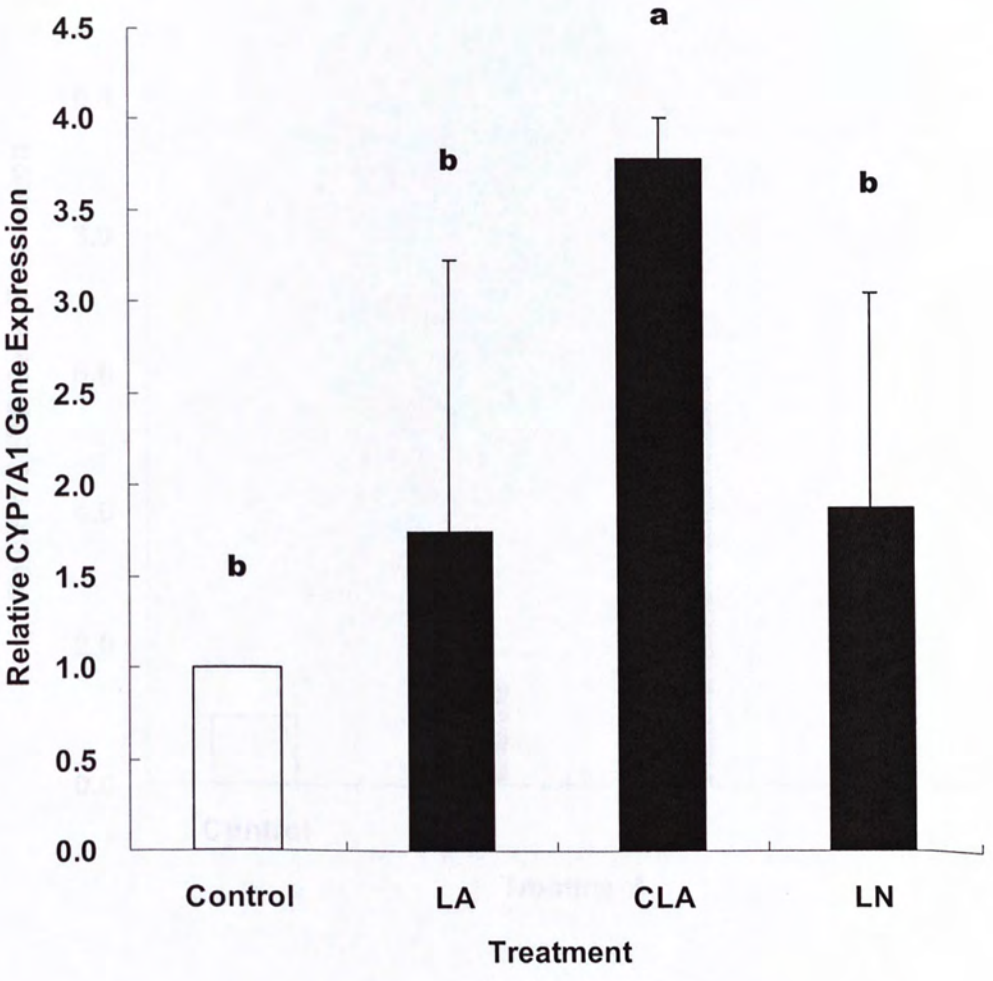

Figure 3.10

Relative CYP7A1 mRNA abundance in HepG2 as determined by real time PCR. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

Means with different superscripts (a, b) differ significantly at $p<0.05$.

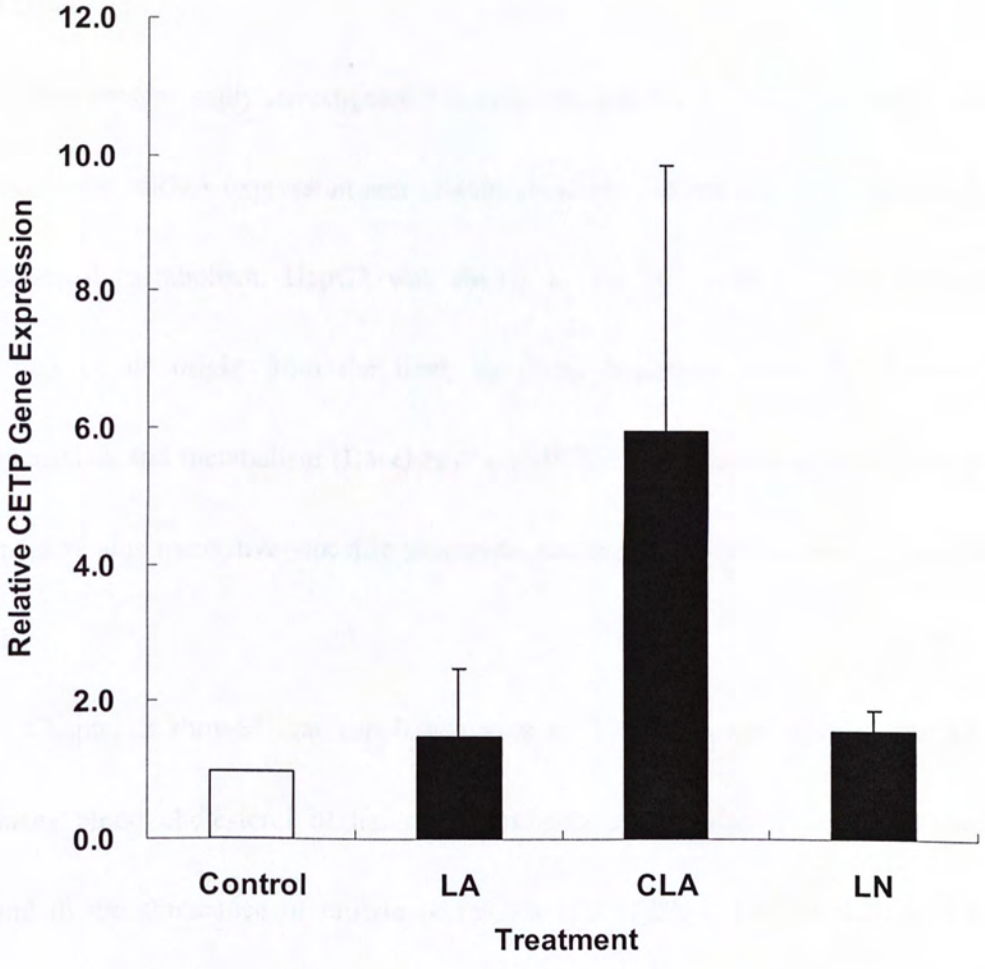

Figure 3.11

Relative CETP mRNA abundance in HepG2 as determined by real time PCR. Data are normalized with β-actin so that value of the control group is regarded as 1.0. Values are expressed as means ± S. D. (n=5).

3.4 Discussion

The present study investigated the effect of LA, CLA or LN in HepG2 cells affected the mRNA expression and protein abundance of several genes involved in cholesterol metabolism. HepG2 was chosen as the cell culture model primarily because of its origin from the liver, the most important organ for cholesterol homeostasis and metabolism (Havekes *et al*, 1987). It has also been established that HepG2 retains many liver-specific properties and that it mimics normal hepatocytes well.

Chapter 2 showed that supplementation of LA, CLA and LN but not CLN reduces blood cholesterol of hamsters. However, no significant differences were found in the abundance of protein or mRNA of SREBP-2, HMGR, LDLR, LXR, CYP7A1 and CETP in their liver. This study was to investigate whether LA, CLA and LN supplementation affect the protein or mRNA levels of these genes using a cell culture model.

The present data suggest that CLA affected the expression of these cholesterol regulating genes distinctly from LA and LN. SREBP-2 mRNA was found to be increased by CLA but not LA or LN. This is in agreement with a previous report that CLA but not LA increased SREBP-2 mRNA in HepG2 (Ringseis *et al* 2006b). However, none of the ODA significantly changed the protein level of SREBP-2.

CLA not LA and LN increased LDLR gene expression but none of them altered LDLR protein abundance. This confirms the report of Yu-Poth *et al* (2004; 2005), who found that CLA but not LA nor LN increased LDLR transcription. Yu-poth *et al* (2004, 2005) also reported no significant difference in the protein of LDLR by LA, CLA and LN.

HMGR mRNA was also increased by CLA but not LA nor LN. This is again in agreement with a report by Ringseis *et al* (2006b), who found up-regulation of HMGR mRNA by CLA but not LA. Since SREBP-2 is the transcription factor for HMGR and LDLR (Eberlé *et al*, 2004) (see Section 1.2.2.2), it is logical that up-regulation of SREBP-2, HMGR and LDLR occur simultaneously. This also suggests that CLA-induced up-regulation of LDLR and HMGR expression is mediated through SREBP-2 transcriptional enhancement.

CLA was shown to increase both LXR protein and mRNA in the present study. In contrast, LN increased LXR mRNA but not its protein, while LA had no effect on both LXR protein and its mRNA. Published literature on the effect of ODA on LXR in HepG2 is limited and offers no comparable data to the present study. However, the simultaneous induction of both protein and mRNA of LXR by CLA provided strong validation.

CYP7A1 induction was observed in cells treated with CLA but LA or LN. There

is also no published report that allows direct comparison with the present data. However, since LXR is known to be the transcription factor of CYP7A1 (see Section 1.2.2.5), it is reasonable that up-regulation of CYP7A1 occurs simultaneously with LXR.

We also observed a trend for increased CETP mRNA by in CLA-treated cells, although the difference was not statistically significant. LA and LN treated cells demonstrated no difference in CETP mRNA level. There is no published data on the effect of CLA on CETP expression, and it should be noted that CETP is a protein which is functional in the blood and hence it carries no biochemical significance in a cell culture system.

The present study observed a significant increase in mRNA level of SREBP-2, LDLR, HMGR and CYP7A1 but not their protein level. Although theoretically, translation into protein occurs after transcription of mRNA and mRNA abundance regulates protein synthesis, it must be noted that two methods of different principles were used in the measurement of protein and mRNA abundances respectively. Protein and mRNA levels were respectively assayed with Western blotting and real-time PCR. There is to date no report that has proven the two methods are of comparable sensitivity. If real-time PCR is more capable of detecting minor differences in mRNA level than Western blotting do in protein level, then significant difference in mRNA

but not in protein might be detected. In fact, since the two methods measures two totally different classes of substances, it would be difficult to compare the sensitivity between the two methods directly. In fact, difference in mRNA but not protein level in LDLR of HepG2 treated with CLA in the absence of oxysterol was also observed in other researchers (Yu-Poth *et al*, 2004).

The above data clearly demonstrated that CLA had a distinctive effect on the cholesterol regulating genes *in vitro* from LA and LN. While LA and CLA have the same number of double bond, the conjugated double bond system in CLA clearly has a different effect on SREBP-2, LDLR, HMGR, LXR and CYP7A1, and hence on cholesterol metabolism compared with the non-conjugated LA and LN. The present data echoes with the previous report of our laboratory that CLA but not LA suppresses intestinal ACAT activity (Yeung *et al*, 2000), showing the distinctive properties of CLA. Recently, there had been reports on the isomer-specific effects of c9, t11 CLA and c10, t12 CLA on some genes (Rasooly *et al*, 2007) and on lipid metabolism in hamsters (Bissonauth *et al*, 2006). The CLA sample (Sigma) we used in this study was a 1:1 mixture of the two. Therefore, it will be interesting to investigate the effect of these two isomers individually on these genes studied.

Chapter 4

Effect of apple polyphenols on blood cholesterol in hamsters

4.1 Introduction

4.1.1 Apple is a commonly consumed fruit worldwide

Apple is the fruit of the *Malus* genus belonging to the rose family *Rosaceae*. The type of apple most commonly consumed nowadays is *Malus domestica* among over 7500 known individual species. Originated in Europe and mid-Asia, it is now cultivated extensively around the world. In 2005, total apple production in Asia and Europe reached 10466 and 15675 kilotonnes respectively (FAO, 2006).

In China, both production and consumption of apples have also increased substantially over the last decade. Annual production of apples increased from 14017 kilotonnes in 1995 to 24017 kilotonnes in 2005, while its annual comsumption had increased from 12602 kilotonnes in 1995 to 19237 kilotonnes in 2005 (FAO, 2006). Apple is becoming a more and more popular fruit in the country.

4.1.2 Potential health effects of apples

Traditionally, apples have been regarded as a healthy food in many cultures, as seen from the popular proverb 'one apple a day keeps the doctor away'. In recent years, association between apple consumption and several health benefits has been

established. Epidemiological studies showed an inverse relationship between apple intake and lung cancer (Feskanich *et al*, 2000; Le Marchand *et al*, 2000), tumors of the alimentary canal (Hertog *et al*, 1994), breast and ovary cancers (Gallus *et al*, 2005), cardiovascular diseases (Sesso *et al*, 2003), diabetes (Song *et al*, 2005) and hypercholesterolemia (Mee & Gee, 1997).

Animal experiments investigating the hypocholesterolemic property of apples in rats was reported as early as in 1968 (Karvinen & Miettinen, 1968). Most of the reported studies investigated either whole lyophilized apples (Aprikian *et al*, 2002; Karvinen & Miettinen, 1968), apple pectin (Aprikian *et al*, 2003; Gonzalez *et al*, 1998) or apple fiber (Mee & Gee, 1997) on cholesterol level. However, there is to date no investigation on the specific effect of apple polyphenols (AP) on blood cholesterol.

4.1.3 Abundance of polyphenols in apple

Apples are rich in phytochemicals, including carotenoids, flavonoids, isoflavonoids and phenolic acids (Boyer & Liu, 2004). The major phenolic compounds in apples are quercetin glycosides, vitamin C, procyanidin, chlorogenic acid, epicatechin and phloretin glycosides (Lee *et al*, 2003). Many of such phytochemicals are strong antioxidants, and their structures are shown in Figure 4.1.

Polyphenols exist in both the flesh and the peel of apples. However, the peel has higher antioxidant activity than the flesh. Apple peel contained two to six times more

phenolic compounds and two to three times more flavonoids than the fresh (Wolfe *et al*, 2003).

4.1.4 Fuji variety of apple

Red Fuji variety of apple was chosen as the raw material for investigation of AP for two reasons: (i) Red Fuji apple is a major apple species produced in China. It accounted for over 64% of total apple harvest in 2005 (Ministry of Agriculture, 2006); and (ii) Fuji apple has been shown to have the highest phenolics content compared with other varieties (Boyer & Liu, 2004).

4.1.5 Objectives

Previous reports on apple's hypocholesterolemic activity investigated either whole apple or apple fiber. The objectives of this study were to investigate the effect of AP on blood cholesterol and cholesterol-regulating enzymes in Golden Syrian hamsters.

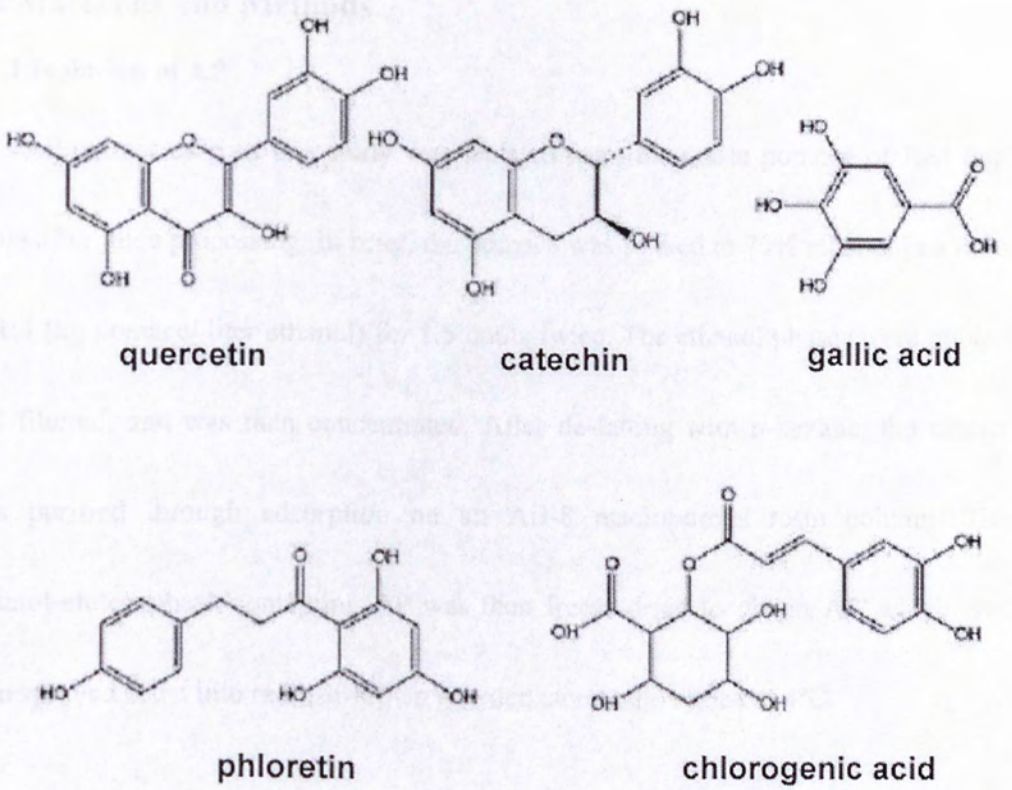

Figure 4.1

Structures of antioxidative phenolic compounds in apples. (Modified from Boyer & Liu, 2004)

4.2 Materials and Methods

4.2.1 Isolation of AP

AP extract used in this study was isolated from the waste pomace of Red Fuji apple after juice processing. In brief, the pomace was soaked in 70% ethanol in a ratio of 1:4 (kg pomace/ liter ethanol) for 1.5 hours twice. The ethanol phases were pooled and filtered, and was then concentrated. After de-fatting with n-hexane, the extract was purified through adsorption on an AB-8 macroporous resin column. The ethanol-eluted phase containing AP was then freeze-dried to obtain AP, which was then sprayed dried into reddish-brown powder, stored and sealed at 4°C.

4.2.2 Characterization of AP extract

Polyphenol content in the AP was determined by HPLC. In brief, AP sample was injected into an HPLC column (Luna C_{18} 100A, 250×4.6mm ID) using a Shimadzu LC-10AT HPLC system equipped with a UV detector. The elution profile was programmed at a flow rate of 1 mL/ min, while the gradient mobile phase composed of 2% acetic acid (solvent A) and 0.4% acetic acid with 80% acetonitrile (solvent B). The ratio of A to B was programmed from 10:1 to 2:8 in 80 minutes and then back to 10:1 in 3 minutes, and then was held for another 22 minutes.

4.2.3 Effect of AP on CETP activity *in vitro*

Effect of apple polyphenol extract on in vitro CETP enzyme activity was studied

using a commercial kit (Amersham Pharmacia Bioscience, Poscataway, NJ, U. S. A.) according to the manufacturer's instructions. Briefly, tritium-labeled HDL and biotinylated LDL were prepared from human plasma. 10μl of ^{3}H-HDL, biotinylated LDL and assay buffer were mixed with 10μl of AP aqueous solutions of various concentrations. The reaction was started by mixing 10μl of a partially purified human CETP and the mixture was incubated at 37°C for 16 hours. After that, a stop solution containing SPA-beads that specifically binded to biotinylated LDL was added to detect ^{3}H radiation emitted from LDL. The reaction mixture was read on a Beckman LS6500 scintillation counter.

This measurement was performed in two trials. In the first one, AP concentrations tested were 0, 0.001, 0.01, 0.1, 1 and 10 mg/ mL. In the second trial, to further study the dose-response pattern, AP concentrations used were 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1 and 0.5 mg/ mL.

4.2.4 Effect of AP on blood cholesterol in hamsters

4.2.4.1 Animals

Thirty-nine (113±6g) male adult Golden Syrian hamsters (*Mesocricetus auratus*) were obtained from the Laboratory Animal Services Centre, The Chinese University of Hong Kong. They were randomly divided into three groups (n=13) and housed (2 or 3 per cage) in wire-bottomed cages at 23°C in a 12-hour light-dark cycle animal

room.

Before the experiment, all the animals were allowed to stabilize by being fed a high fat high cholesterol diet (control diet). From week 0 to week 6, one group were continued to fed the control diet and the other two group were fed a similar diet except that they were respectively supplemented with 0.3% (0.3AP) and 0.6% (0.6AP) AP polyphenol extract. During the study, food was given daily and any uneaten food was discarded. The amount of food consumed was measured each day. The animals were free to access to food and distilled water and were weighed weekly.

Blood (1 mL) was bled from the retro-orbital sinus from the animals into a heparinized capillary tube at the end of weeks 0, 3 and 6 after food deprivation for 16 hours. The blood was centrifuged at 3000rpm for 10 minutes and the plasma was collected and stored at -20 °C until analysis.

The last blood sample was collected on the end of week 6. After a 3-day recovery, all hamsters were sacrificed by nitrogen suffocation without fasting. Blood was collected from abdominal artery into a vacuum heparinized tube, centrifuged at 800 × g at 10 minutes and the plasma was collected and stored at -20°C until analysis. The liver, heart, kidney, adipose tissue (perirenal and epididymal pads) were removed, washed in saline, weighed, flash frozen in liquid nitrogen and stored at -80°C until analysis.

4.2.4.2 Diets

The control diet was prepared by mixing the following ingredients in proportion (g/kg diet): cornstarch, 508; casein, 242; lard, 50; sucrose, 119; mineral mix AIN-76, 40; viamin mix AIN-76A, 20; DL-methionine, 1; cholesterol, 1. The two experimental diets were prepared by adding 0.3% and 0.6% (w/w) extracts into the control diet respectively. The powered diets were mixed with a gelatin solution (20g/L) in a ratio of 200g diet per liter of solition. (Table 4.1) Once the gelatin has set, the diets were cut into pieces of approximately 10 g cubes and stored forzen at -20°C.

4.2.4.3 Plasma lipids measurement

Plasma cholesterol and triglyceride were measured by commercial enzymatic kits from Thermo and Stanbio respectively according to the manufacturer's instructions. For measurement of plasma HDL-cholesterol, LDL and VLDL were first precipitated with phosphotungstic acid and magnesium chloride in a commerical kit (Sigma). Non-HDL cholesterol was calculated by deducting HDL-cholesterol from total cholesterol.

Table 4.1

Composition of the control and two apple polyphenol (AP) diets (g)

	Control	**0.3 AP**	**0.6 AP**
Cornstarch	508	508	508
Casein	242	242	242
Lard	50	50	50
Sucrose	119	119	119
Mineral mix AIN-76	40	40	40
Vitamin mix AIN-76A	20	20	20
DL-methionine	1	1	1
Cholesterol	1	1	1
Apple polyphenol extract	**0**	**3**	**6**
Gelatin	20	20	20

4.2.4.4 Plasma CETP activity measurement and immunoreactive mass by Western blotting

The plasma CETP acitivity of the hamsters were measured as previously described in section 2.2.1.5, Chapter 2. For measurement of CETP immunoreactive mass, plasma was diluted in a buffer containing 20 mM Tris-HCl (pH 7.5), 2mM $MgCl_2$, 0.2 M sucrose and protease inhinitor cocktail pellet (Complete®, Roche ®, Mannheim, Germany). The diluted sample (0.01μL) was size-separated on 7% SDS-PAGE at 120V for 2 hours. After electrophoresis, the proteins were transferrred on a Hybond-P PVDF membrane (Amersham Pharmacia Biosciences) with a semi-dry transfer cell (Bio-rad) at 15 V for 1 hour. The membrane was blocked in a blocking solution (1X Tris-buffered saline, 0.1% Tween-20 and 5% non-fat milk) at 4°C for 1 hour and then overnight at 4°C in the same solution containing 1:600 anti-CETP antibody (Abcam plc, Cambridge, UK). The membrane was then washed three times for 10 minutes and then incubated in the blocking solution containing 1:3000 horseradish peroxidase-linked anti-rabbit IgG (Abcam plc, Cambridge, UK) at 4°C for 1 hour. The washes were repeated before the membranes were developed with ECL chemiluminescent agent (Amersham Life Science) and subjected to autoradiography for one to five minutes on SuperRX medical X-ray film (Fuji). Band size and optical density were quantified using the computer software Photoshop® (Adobe Systems Inc, CA, USA).

4.2.4.5 Measurement of liver SREBP-2, LDL-R, HMG-R and CYP7A1 protein abundance by Western blotting

Hepatic protein extraction and Western blotting analysis of liver SREBP-2, LDLR, HMGR and CYP7A1 were carried out as previously described in section 2.2.1.6, Chapter 2.

4.2.4.6 Statistics

Results were presented as means± standard deviation (S.D.). Where applicable, statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by Student's *t*-test, using Prism® (Graphpad software, Inc., CA, U. S. A.). Differences between groups were considered significant when $P<0.05$.

4.3 Results

4.3.1 Polyphenol content in AP

A typical HPLC diagram is shown on Fig. 4.2. The peaks identified included chlorogenic acid, phloridzin, catechin and epicatechin.

4.3.2 Effect of AP on CETP activity *in vitro*

Transfer of ^{3}H-cholesterol from HDL to LDL decreased as AP concentration increased. In the first trial, CETP activity dropped significantly at AP concentration higher than 0.01 mg/ mL (Figure 4.3A). In the second trial, significant decrease in ^{3}H-cholesterol transfer occurred at AP concentrations higher than 0.02 mg/ mL (Figure 4.3B).

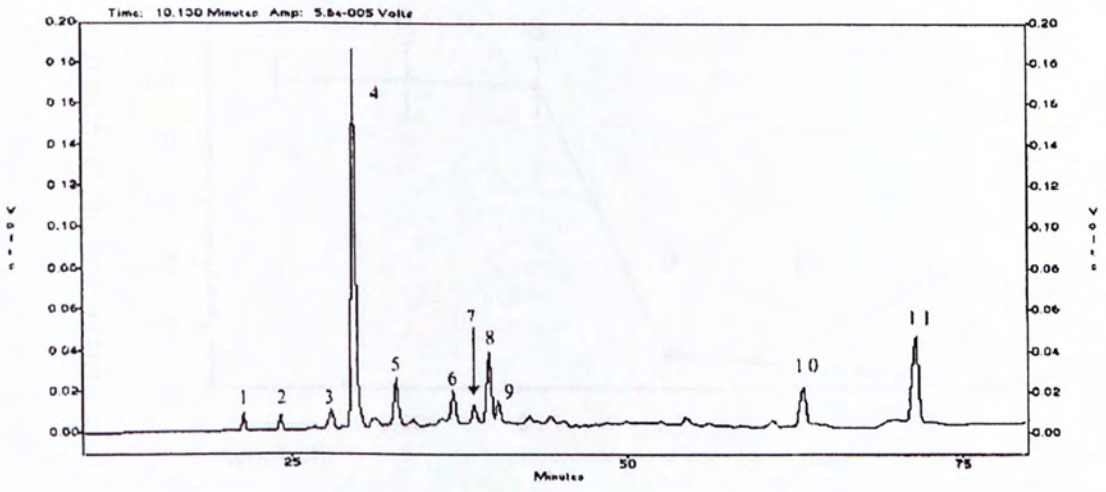

Figure 4.2

HPLC chromatogram of apple polyphenols (AP) prepared from Red Fuji Apples. For AP extraction process, see 4.2.1. For HPLC profile conditions, see 4.2.2. Identification of peaks: 3=catechin; 4=chlorogenic acid; 6=epicatechin; 11=phloridzin. Peaks 1, 2, 5, 7, 8, 9, 10=unknown.

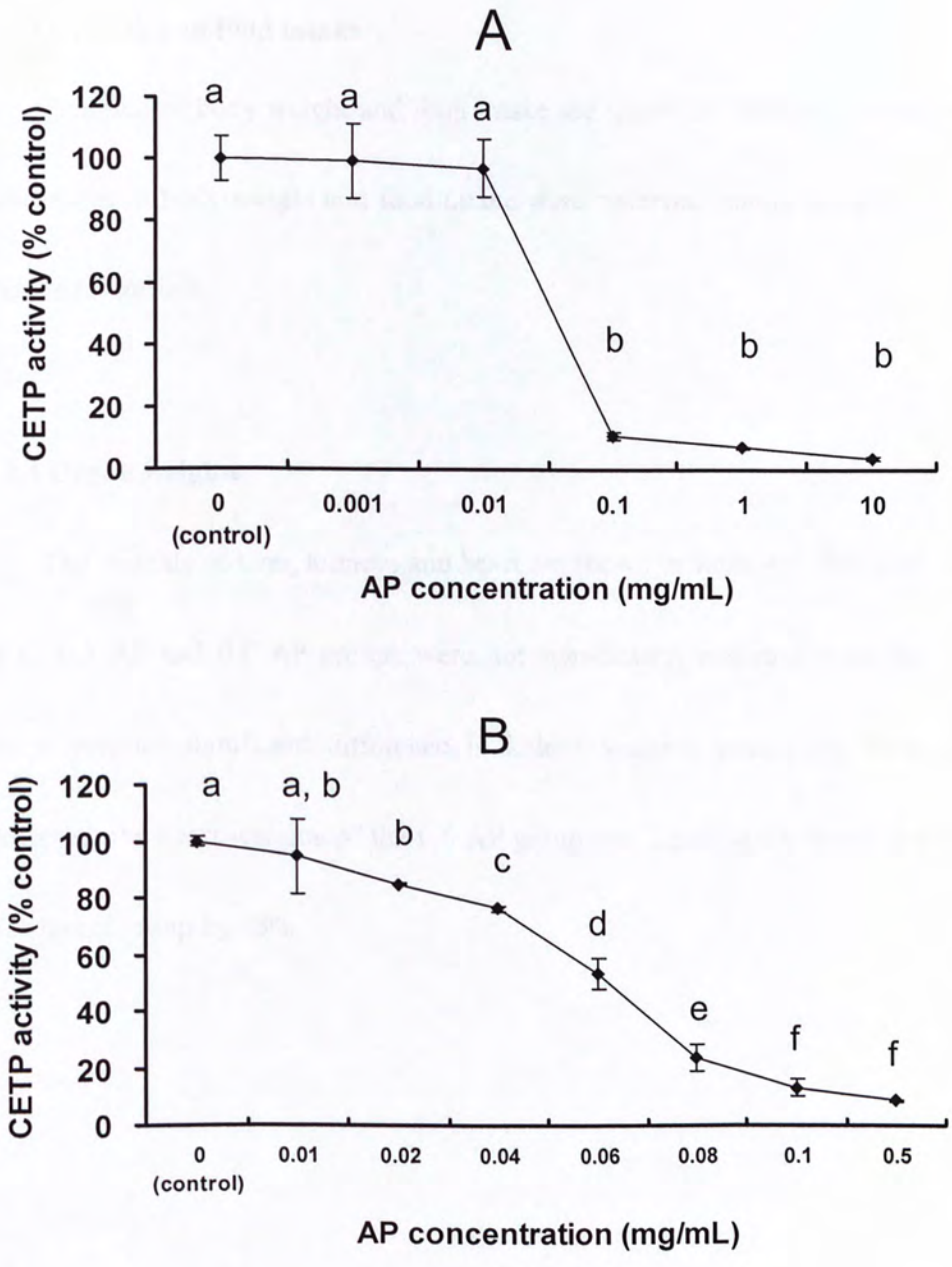

Figure 4.3

Concentration-response curves of AP on in vitro CETP activity. (A) The first trial: CETP activity decreased at AP concentrations higher than 0.01 mg/mL. (B) The second trial: effect of AP concentration ranged 0-0.5 mg/mL on CETP activity. Data are normalized so that CETP activity of the control group is regarded as 100%. Values are expressed as means ± SD (n=3). Values with different letters (a, b, c, d, e, f) are significantly different from that of their control at $p<0.05$.

4.3.3 Growth and food intake

The gain of body weight and food intake are shown in Table 4.2. No significant differences in body weight and food intake were observed among the control, 0.3AP and 0.6AP groups.

4.3.4 Organ weights

The weights of liver, kidneys and heart are shown in Table 4.3. The liver weights of of 0.3 AP and 0.6 AP groups were not significantly different from the control. There was no significant difference in kidney weights among the three groups. However, the heart weights of the 0.6 AP group was significantly lower than that of the control group by 13%.

Table 4.2

Body weight gain and food intake of the hamsters

	Control	0.3 AP	0.6 AP
Initial body weight (g)	113.5±5.5	115.8±4.5	111.2±7.2
Final body weight (g)	120.8±10.0	120.8±7.7	114.2±7.9
Food intake (g/day)	10.3±1.4	10.2±1.4	9.7±1.4

Values are expressed as mean ± S.D. (n=13).

Table 4. 3

Weights of liver, kidney and heart in hamsters fed the control, 0.3 AP and 0.6 AP diets

	Control	0.3 AP	0.6 AP
Liver (g)	$6.59 \pm 0.86^{a, b}$	7.00 ± 0.76^{a}	6.29 ± 0.51^{b}
Kidneys (g)	1.12 ± 0.11	1.18 ± 0.07	1.12 ± 0.08
Heart (g)	0.55 ± 0.10^{a}	$0.53 \pm 0.09^{a, b}$	0.48 ± 0.06^{b}

Values are expressed as mean ± S.D. (n=13).

Means at the same row with different superscripts (a, b) differ significantly at $p<0.05$.

4.3.5 Effect of AP supplementation on the plasma lipid profile of hamsters

Plasma TC in hamsters fed AP diets was not significantly different from that of the control. However, plasma HDL-C of the AP-fed hamsters was found to be significantly higher than that of the control (Table 4. 4). To be specific, HDL-C of 0.3 AP and 0.6 AP groups was 14.7% and 16.5% higher than that of the control, respectively. In contrast, nHDL-C in the 0.3 AP and 0.6 AP groups were significantly lower than the control by 20.0% and 36.7% respectively, leading to a lower ratio of nHDL-C to HDL-C in the AP fed groups than in the control (Table 4. 4).

Plasma TG in the 0.3 AP group was not significantly different from the control but that of the 0.6 AP group was significantly lower than the control by 31.9% (Table 4. 4).

4.3.6 Effect of AP feeding on plasma CETP activity of the hamsters

Plasma CETP activity in hamsters fed the AP diets was significantly lower than that of the control. To be specific, plasma CETP activity of the 0.3 AP and 0.6 AP groups was 71.7% and 70.1% of the control (Figure 4.4).

Table 4. 4

Effect of AP feeding on plasma triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), non-HDL cholesterol (nHDL-C) and the ratio of nHDL-C to HDL-C in the hamsters.

	Control	0.3 AP	0.6 AP
TC (mg/ dL)	199 ± 28	197 ± 26	184 ± 12
HDL-C (mg/ dL)	109 ± 18^{b}	125 ± 15^{a}	127 ± 13^{a}
LDL-C (mg/ dL)	90 ± 13^{a}	72 ± 15^{b}	57 ± 11^{c}
nHDL-C/ HDL-C (mg/ dL)	0.84 ± 0.12^{a}	0.58 ±0.10^{b}	0.46 ± 0.11^{c}
Triglycerides (mg/ dL)	144 ± 31^{a}	142 ± 45^{a}	98 ± 28^{b}

Values are expressed as mean ± S. D. (n=13). nHDL-C was calculated by subtracting HDL-C from TC.

Means at the same row with different superscripts (a, b, c) differ significantly at $p<0.05$.

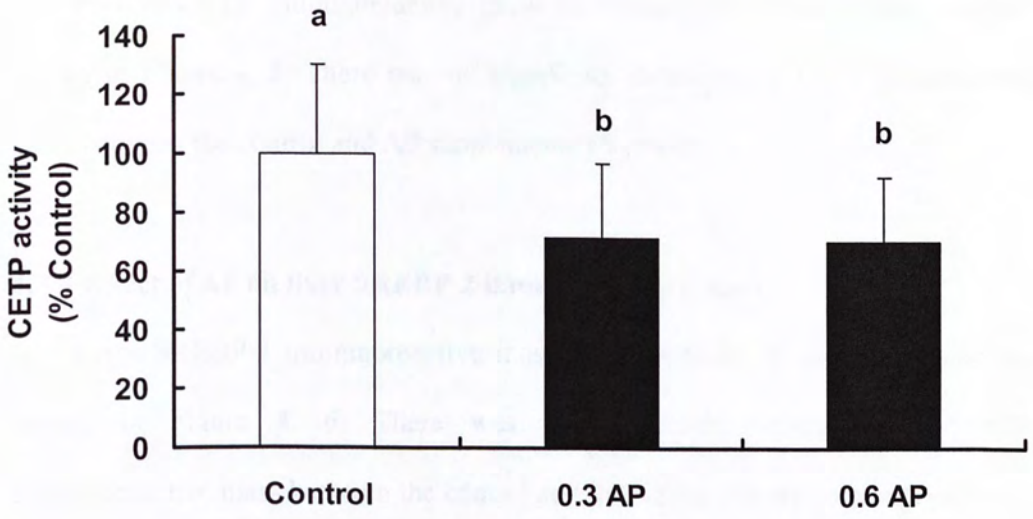

Figure 4.4

Plasma CETP activity in the hamsters fed the control and AP-supplemented diets. Hamsters fed both 0.3% and 0.6% AP diets had significantly reduced plasma CETP activity than the control. Data are normalized so that CETP activity of the control group is regarded as 100%. Values are expressed as means ± S.D. (n=13). Means with different superscripts (a, b) differ significantly at $p<0.05$.

4.3.7 Effect of AP on plasma CETP immunoreactive mass

Plasma CETP Immunoreactive mass as revealed by Western Blot analysis is shown in Figure 4. 5. There was no significant difference in CETP immunoreative mass between the control and AP supplemented hamsters.

4.3.8 Effect of AP on liver SREBP-2 immunoreactive mass

Liver SREBP-2 immunoreactive mass as revealed by Western Blot analysis is shown in Figure 4. 6. There was no significant difference in SREBP-2 immunoreactive mass between the control and the AP supplemented hamsters.

4.3.9 Effect of AP on liver LDLR immunoreactive mass

Liver LDLR immunoreactive mass as revealed by Western Blot analysis is shown in Figure 4. 7. There was no significant difference in LDLR immunoreactive mass between the control and the AP supplemented hamsters.

4.3.10 Effect of AP on liver HMGR immunoreactive mass

Liver HMGR immunoreactive mass as revealed by Western Blot analysis is shown in Figure 4. 8. There was no significant difference in HMGR immunoreactive mass between the control and the AP supplemented hamsters.

4.3.11 Effect of AP on liver CYP7A1 immunoreactive mass

Liver CYP7A1 immunoreactive mass as revealed by Western Blot analysis is shown in Figure 4. 9. There was no significant difference in CYP7A1 immunoreactive mass between the control and the AP supplemented hamsters.

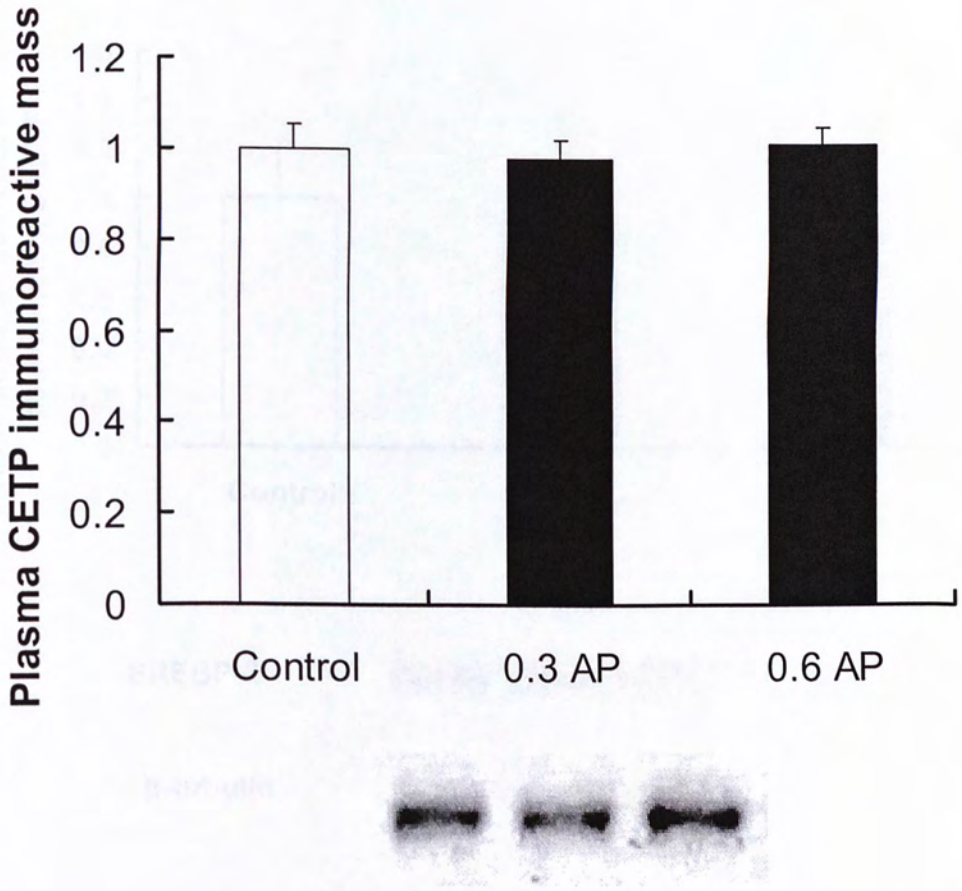

Figure 4.5

CETP immunoreactive mass measured by Western Blotting. Data are normalized so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=13).

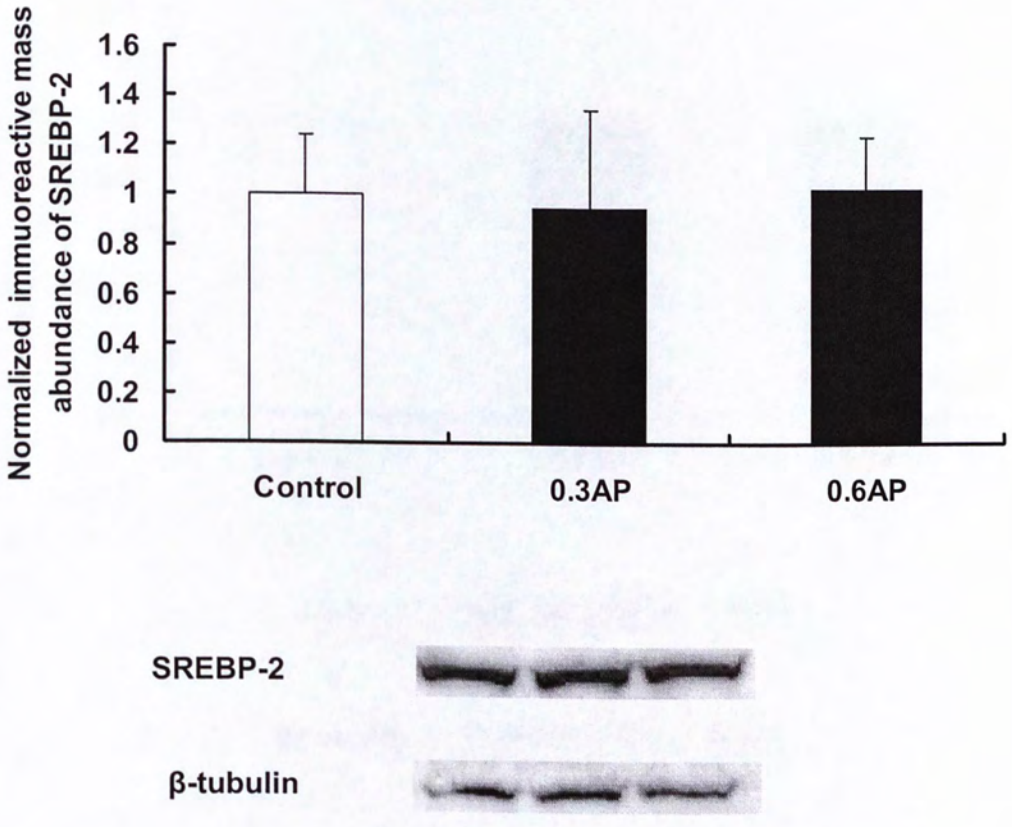

Figure 4. 6

Relative abundance of hepatic SREBP-2 immunoreactive mass as determined by Western Blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=13).

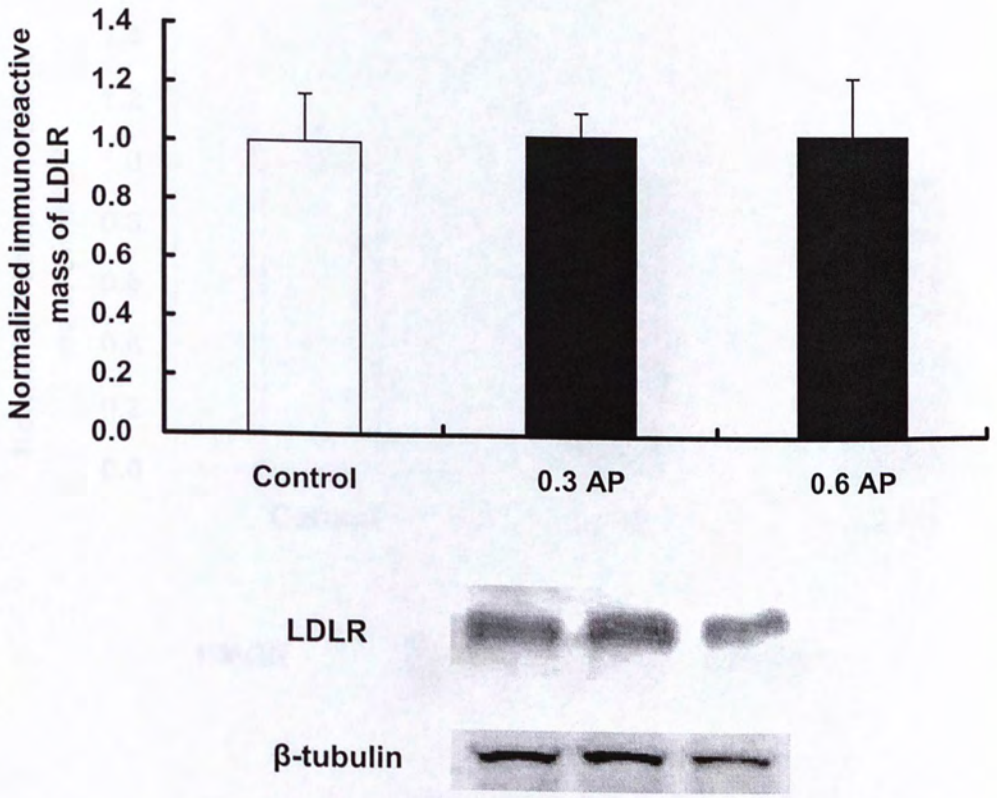

Figure 4. 7.

Relative abundance of hepatic LDLR immunoreactive mass as determined by Western Blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=13).

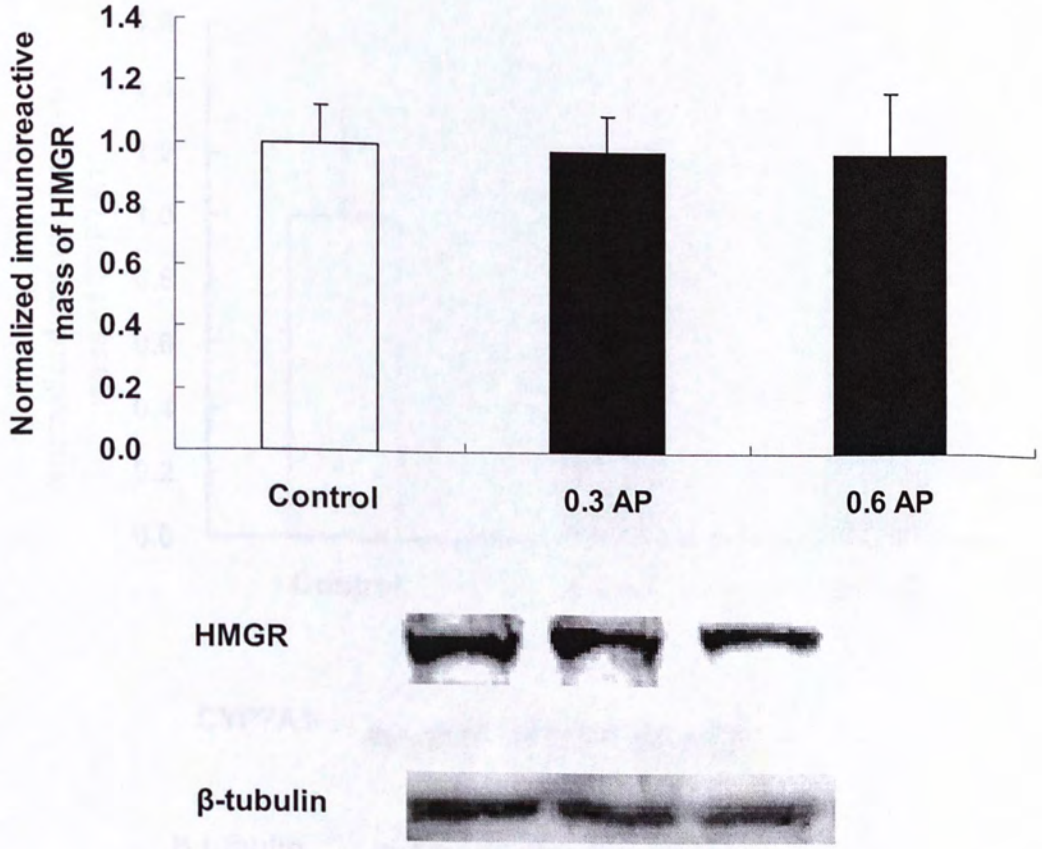

Figure 4. 8.

Relative abundance of hepatic HMGR immunoreactive mass as determined by Western Blot analysis. Data are normalized with β-tubulin so that value of the control group is treated as 1.0. Values are expressed as means ± S.D. (n=13).

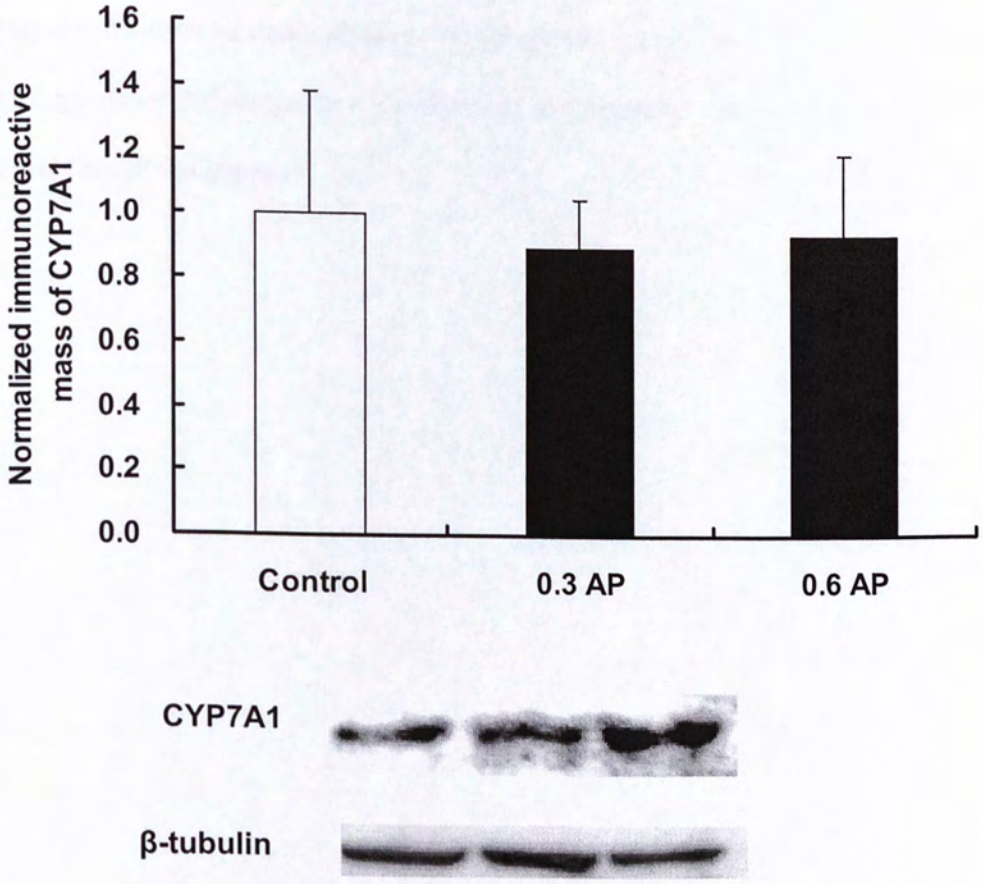

Figure 4. 9

Relative abundance of hepatic CYP7A1 immunoreactive mass as determined by Western Blot analysis. Data are normalized with β-tubulin so that value of the control group is regarded as 1.0. Values are expressed as means ± S.D. (n=13).

4.3.12 Effect of AP on liver cholesterol level

Hepatic cholesterol concentrations of the animals are shown Figure 4. 10. There was no significant difference in cholesterol concentrations in the liver of the control group and the AP fed groups.

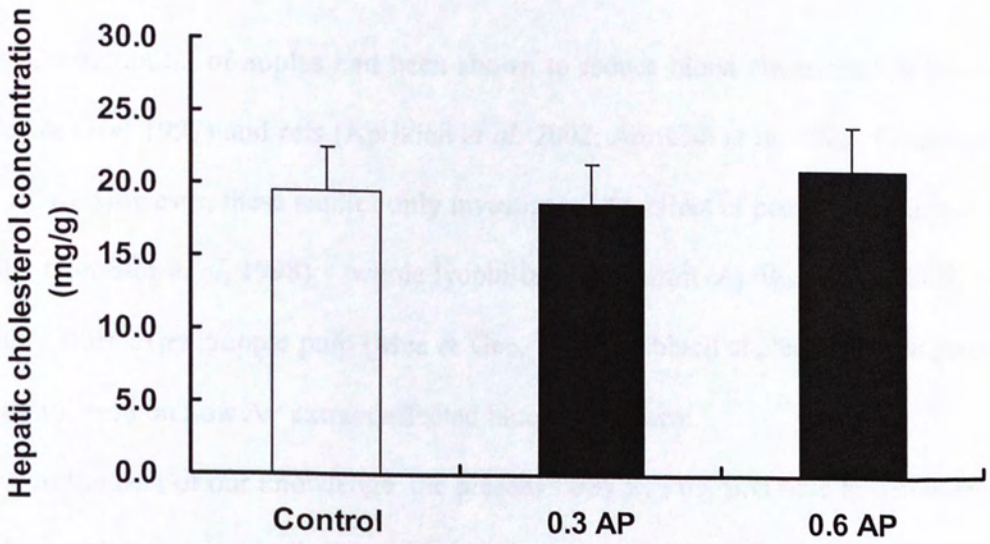

Figure 4. 10

Hepatic cholesterol concentration in the hamsters as determined by GC. Values are expressed as means ± S.D. (n=13).

4.4 Discussion

Consumption of apples had been shown to reduce blood cholesterol in humans (Mee & Gee, 1997) and rats (Aprikian *et al*, 2002; Aprikian *et al*, 2003; Gonzalez *et al*, 1998). However, these studies only investigated the effect of pectin (Aprikian *et al*, 2003; Gonzalez *et al*, 1998), whole lyophilized apple fruit (Aprikian *et al*, 2002) and dietary fiber extract/apple pulp (Mee & Gee, 1997) on blood cholesterol. The present study focused on how AP extract affected blood cholesterol.

To the best of our knowledge, the present study was the first time to demonstrate that supplementation of AP did not affect plasma TC, but it increased HDL-cholesterol, resulting in a reduced nHDL-C/HDL-C ratio (Table 4.4). This is in agreement with a study on Wistar rats by Nakazato (2006) who reported that apple polyphenols had no hypocholesterolemic activity, but they did not measure cholesterol distribution in different lipoprotein classes. In another study, consumption of apple juice was found to have no significant effect on blood TC or HDL-C in children (Dennison *et al*, 1999). The discrepancy from the present finding might be because they used human children as subjects and apple juice instead of AP extract was studied.

The present study also demonstrated that AP supplementation reduced CETP activity both *in vitro* and *in vivo*. As shown in Figure 4.3, at sufficiently high concentrations, AP acted as an inhibitor to CETP *in vitro* and such property was confirmed *in vivo,* finding that AP-fed hamsters had CETP activity lower than the control group (Figure 4.4). Western Blotting showed no difference in plasma CETP immunoreactive mass between the AP-fed and control hamsters, suggesting that AP did not affect expression of CETP protein in hamsters. This clearly showed that AP did not regulate translation of CETP gene but it inhibited its enzyme activity in

plasma.

In recent years, inhibiting CETP activity as a strategy of raising blood HDL cholesterol has become a popular investigation topic (Barter & Kastelein, 2006; Doggrell, 2006; Dullaart *et al*, 2007; Klerkx *et al*, 2006; Okamoto *et al*, 2007; Shah, 2007; Van der Steeg *et al*, 2005). The present study is in agreement with those of Kothari (1997) and Rittershaus (2000) who showed that inhibition of CETP activity was associated with an increase in blood HDL-cholesterol level. Elevated HDL-cholesterol has been shown to be associated with lower risks of cardiovascular diseases and myocardial infarctions (Pischon *et al*, 2005). Both of the AP-fed groups had lower non-HDL-cholesterol to HDL-cholesterol ratio, suggesting that AP modified lipoprotein profile favorably against atherosclerosis (Kawamoto *et al*, 2005).

Previous studies reported that whole apple fruit, apple fiber and apple pectin reduced TC in animals (Gonzalez *et al*, 1998; Karvinen & Miettinen, 1968; Leontowicz *et al*, 2003) but apple polyphenol extract alone did not affect TC level in rats (Nakazato *et al*, 2006). Together with the present study, the fiber and pectin in apple could reduce the total cholesterol while AP could affect the distribution of cholesterol among lipoprotein classes. In other words, apples contain at least two classes of substances that could improve blood cholesterol independently.

The present study did not find evidence that AP could affect SREBP-2, LDL-R, HMGR and CYP7A1, at both transcriptional and translational levels. It is unknown how AP affects plasma CETP activity. It was reported that heavy alcohol consumption reduced carbohydrate content in CETP, leading to a defected CETP (Liinamaa *et al*, 2006). Whether AP affects plasma CETP activity in the same way is not known. It should be noted that polyphenols undergo significant structural modification once they are absorbed into enterocytes and in the liver by cytochrome P450 enzymes

(Kroon *et al*, 2004). It deems necessary to investigate the chemical form of apple polyphenols and to investigate their effects on CETP glycosylation.

Chapter 5
Conclusion

Arterial cholesterol deposition due to hypercholesterolemia has been established to closely associate with the development of atherosclerosis. Dietary PUFAs and phytochemicals have been shown to improve blood lipid profile and slow down atherosclerosis progression.

LA, CLA, LN and CLN are four structurally similar ODA. The present study demonstrated that only incorporation of 2% (w/w) LA, CLA and LN but not CLN in the diet was hypocholesterolemic in hamsters. Blood TC was decreased by both LA and CLA feeding, but non-HDL cholesterol was reduced to a larger extent by CLA than LA, resulting in a significantly lower non-HDL cholesterol/ HDL cholesterol ratio. In contrast, LN but not CLN reduced blood total cholesterol and non-HDL cholesterol. Hepatic cholesterol level was significantly reduced by the four ODA, with CLA reduced the most, followed by LN, CLN and LA. It was concluded that double bond conjugation on LA enhances its hypocholesterolemic activity but this has an opposite effect for LN.

No significant difference was found in hepatic protein of SREBP-2, HMGR, LDLR, LXR and CYP7A1. No significant difference was found in hepatic mRNA of CETP, LDLR, LXR and CYP7A1 either. Plasma CETP activity was not significantly affected in hamsters fed ODA. However, cholesterol balance study clearly demonstrated that cholesterol retention was significantly decreased by LA, CLA and LN, but not CLN. Intestinal ACAT activities of CLA and LN, but not LA and CLN-fed hamsters, were significantly lower than the control. It was concluded that CLA and LN but not CLN lowered blood total and non-HDL cholesterol through

inhibition on intestinal ACAT, leading to reduced cholesterol absorption, but had no effect on hepatic SREBP-2, HMGR, LDLR, LXR and CYP7A1 at both transcriptional and translational levels.

Supplementation of 0.4 mmol/L of CLA in the medium up-regulated gene expressions of SREBP-2, HMGR, LDLR, LXR and CYP7A1 in HepG2. However, significant increase in protein abundance was only found in LXR but not SREBP-2, HMGR, LDLR or CYP7A1 in CLA treated cells. Supplementation of LA and LN did not significantly affect the protein abundance of SREBP-2, HMGR, LDLR, LXR and CYP7A1. It was concluded that CLA up-regulated gene expression of SREBP-2, HMGR, LDLR, LXR and CYP7A1 and protein abundance of LXR distinctively over LA and LN *in vitro*.

Hypocholesterolmic effect of AP on CETP activity was studied *in vitro* and *in vivo*. It was demonstrated that AP inhibited CETP activity in a dose dependent manner *in vitro*. AP concentration of 0.02 mg/ mL or above was found to significantly inhibit transfer of cholesterol from HDL to LDL. We then investigated the effect of AP on blood cholesterol in hamsters, and found that supplementation of 0.3% or 0.6% of AP in the diet did not affect blood TC, but it increased HDL-cholesterol and decreased LDL-cholesterol, leading to a reduced non-HDL cholesterol/ HDL-cholesterol ratio. TG level was also significantly reduced in hamsters fed 0.6% AP. No significant difference was found in hepatic cholesterol level in AP fed hamsters. Protein abundance of plasma CETP and hepatic SREBP-2, LDLR, HMGR and CYP7A1 was not significantly different in the hamsters. However, plasma CETP activity was significantly reduced in both 0.3% and 0.6% AP-fed hamsters. Since no difference in plasma CETP protein level was detected, and *ex vivo* inhibition of CETP by AP was found, the inhibition was estimated to occur at a post-translational level. It was

concluded that AP had no effect on blood TC but favorably improved distribution of cholesterol in lipoproteins through direct inhibition on CETP.

The activity of LA, CLA, LN and AP to favorably change the blood lipid profile was confirmed in the present study. However, only hamsters and *in vitro* models were used to test the activity of ODA and AP on blood cholesterol. There is no information whether or how our findings apply to humans. Based on the present results, it deems necessary to further investigate the effect of ODA and AP on cholesterol metabolism in human subjects.

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